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Methods of using an AL-1 neurotrophic factor immunoadhesin

Abstract:

The present invention provides nucleic acids encoding AL-1 protein, as well as AL-1 protein produced by recombinant DNA methods. Such AL-1 protein is useful in preparing antibodies and in diagnosing and treating various neuronal disorders.

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<b>(21) International Application Number:</b> PCT/US95/14016 <b>(22) International Filing Date:</b> 26 October 1995 (26.10.95)  <b>(30) Priority Data:</b> 08/330,128 27 October 1994 (27.10.94) US 08/486,449 7 June 1995 (07.06.95) US  <b>(60) Parent Application or Grant</b> <b>(63) Related by Continuation</b> US 08/486,449 (CIP) Filed on 7 June 1995 (07.06.95)  <b>(71) Applicant (for all designated States except US):</b> GENENTECH, INC. [US/US]; 460 Point San Bruno Boulevard, South San Francisco, CA 94080-4990 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> CARAS, Ingrid, W. [GB/US]; 456 Hoffman Avenue, San Francisco, CA 94114 (US). WINSLOW, John, W. [US/US]; 954 Ventura Street, El Granada, CA 94018 (US).	<b>(74) Agents:</b> TORCHIA, Timothy, E. et al.; Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080-4990 (US).  <b>(81) Designated States:</b> AL, AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, LS, MW, SD, SZ, UG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
<b>(54) Title:</b> AL-1 NEUROTROPHIC FACTOR, A LIGAND FOR AN EPH-RELATED TYROSINE KINASE RECEPTOR  <b>(57) Abstract</b>  The present invention provides nucleic acids encoding AL-1 protein, as well as AL-1 protein produced by recombinant DNA methods. Such AL-1 protein is useful in preparing antibodies and antagonists and in diagnosing and treating various neuronal disorders and disorders or conditions associated with angiogenesis.		

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# AL-1 neurotrophic factor, a ligand for an EPH related tyrosine kinase receptor

## Field of the Invention

This application relates to the production of polypeptides involved in neuronal survival and/or growth and in angiogenesis, in particular the production of purified forms thereof by means of recombinant DNA technology.

## Background of the Invention

A number of protein neurotrophic factors, or neurotrophins, have been identified which influence growth and development of the vertebrate nervous system. It is believed that these factors play an important role in promoting the differentiation, survival, and function of diverse groups of neurons in the brain and periphery.

The belief that neurotrophic factors have important signalling functions in neural tissues is based upon the precedent established by work with nerve growth factor (NGF). NGF has been shown, both *in vitro* and *in vivo*, to support the survival of sympathetic, sensory, and basal forebrain neurons. Administration of exogenous NGF rescues neurons from cell death during development. Conversely, removal or sequestration of endogenous NGF by administration of anti-NGF antibodies promotes such cell death. Heumann, *J. Exp. Biol.* 132:133-150 (1987); Hefli, *J. Neurosci.* 6:2155-2162 (1986); Thoenen and Barde, *Annu. Rev. Physiol.* 60:284-335 (1980).

Additional neurotrophic factors related to NGF have since been identified. These include brain-derived neurotrophic factor (BDNF) (Leibrock, et al., *Nature* 341:149-152 (1989)), neurotrophin-3 (NT-3) (Kaisho, et al., *FEBS Lett.* 266:187 (1990); Maisonpierre, et al., *Science* 247:1446 (1990); Rosenthal, et al., *Neuron* 4:767 (1990)), and neurotrophin 4/5 (NT-4/5) (Berkmeier, et al., *Neuron* 7:857-866 (1991)).

Neurotrophins, similar to other polypeptide growth factors, affect their target cells through interactions with cell surface receptors. According to our current understanding, two kinds of transmembrane glycoproteins act as receptors for the known neurotrophins. Equilibrium binding studies have shown that neurotrophin-responsive neuronal cells possess a common low molecular weight (65,000 - 80,000 Daltons), low affinity receptor, typically referred to as p75<sup>NGFR</sup> or p75, and high molecular weight (130,000 - 150,000 Daltons), high and low affinity receptors that are members of the trk family of receptor tyrosine kinases.

Receptor tyrosine kinases are known to serve as receptors for a variety of protein factors that promote cellular proliferation, differentiation, and survival. In addition to the trk receptors, examples of other receptor tyrosine kinases include the receptors for epidermal growth factor (EGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF). Typically, these receptors span the cell membrane, with one portion of the receptor being intracellular and in contact with the cytoplasm, and another portion of the receptor being extracellular. Binding of a ligand to the extracellular portion of the receptor is believed to induce tyrosine kinase activity in the intracellular portion of the receptor, with ensuing phosphorylation of various intracellular proteins involved in cellular signalling pathways.

In addition to receptor tyrosine kinases that serve as receptors for known protein factors, many receptor-like tyrosine kinases have been identified for which no ligand is known. Examples of such "orphan"

receptors include recently discovered members of the eph family, which includes eph, elk, cek5, cek7, mek4/cek4/hek, sek, hek2, and bsk (Tuzi, et al., Br. J. Cancer 69:417-421 (1994); Zhou, et al., J. Neurosci. Res. 37:129-143 (1994)). Recently, other eph family receptor ligands have been reported including B61, an Eck receptor ligand (Bartley, et al., Nature 368:558-560 (1994) and Pandey et al. Science (1995) 268:567-569),  
5 ELF-1, a Mek4 and Sek receptor ligand (Cheng et al. Cell (1995) 82:371-381), Ehk-1-2, LERK2, HTK-L and CEK5-L. After the discovery reported in the present application was made, a chicken AL-1 homolog termed RAGS was reported (Drescher et al. Cell (1995) 82:359-370).

Although eph family members are expressed in many different tissues, several family members are expressed in the nervous system or specifically in neurons (Maisonpierre, et al., Oncogene 8:3277-3288 (1993);  
10 Lai, et al., Neuron 6:691-704 (1991). In order to better understand the role of these and other orphan receptor tyrosine kinases in the nervous system, it would be useful to identify new ligands that bind to such receptors.

The present invention is based on successful research resulting in the identification, cloning, and sequencing of an eph-related tyrosine kinase receptor, referred to as REK7, and its ligand, referred to as AL-1.

It is an object of the present invention to provide nucleic acid encoding AL-1, and to use the nucleic  
15 acid to produce AL-1 in recombinant host cells for diagnostic use or for therapeutic use.

It is another object to use such nucleic acids encoding AL-1, and portions thereof, to identify related nucleic acids in the cells or tissues of various animal species.

It is another object to provide derivatives and modified forms of AL-1, including amino acid sequence variants and covalent derivatives thereof, as well as antagonists of AL-1. It is another object to prepare  
20 immunogens for raising antibodies, as well as to obtain antibodies, capable of binding to AL-1, or derivatives or modified forms thereof.

These and other objects of the invention will be apparent to the ordinary artisan upon consideration of the specification as a whole.

#### Summary of the Invention

25 These objects are accomplished by first providing isolated DNA comprising the nucleotide coding sequence for AL-1, an expression vector comprising the nucleotide coding sequence for AL-1, host cells transformed with the vector, including mammalian and bacterial host cells, and a method of using a nucleic acid molecule encoding AL-1 to effect the production of AL-1, comprising culturing a host cell transfected to express such nucleic acid molecule and recovering AL-1 from the host cell culture. In this method, preferably the host  
30 cell is transfected with an expression vector comprising the nucleotide coding sequence for AL-1.

By providing the full nucleotide coding sequence for AL-1, the invention enables the production of AL-1 by means of recombinant DNA technology, thereby making available for the first time sufficient quantities of substantially pure AL-1 protein or AL-1 antagonists for diagnostic and therapeutic uses with a variety of neurological disorders and with diseases and conditions that are angiogenesis-dependent such as solid tumors,  
35 diabetic retinopathy, rheumatoid arthritis, and wound healing.

In a preferred embodiment, the invention provides AL-1 that is free of other human proteins.

Modified and variant forms of AL-1 are produced *in vitro* by means of chemical or enzymatic treatment or *in vivo* by means of recombinant DNA technology. Such polypeptides differ from native AL-1, for example, by virtue of one or more amino acid substitutions, deletions or insertions, or in the extent or pattern of glycosylation, but substantially retain a biological activity of native AL-1.

5        Antibodies to AL-1 are produced by immunizing an animal with AL-1 or a fragment thereof, optionally in conjunction with an immunogenic polypeptide, and thereafter recovering antibodies from the serum of the immunized animals. Alternatively, monoclonal antibodies are prepared from cells of the immunized animal in conventional fashion. Antibodies obtained by routine screening will bind to AL-1 but will not substantially bind to (i.e., cross react with) NGF, BDNF, NT-3, NT-4/5, or other neurotrophic factors. Immobilized anti-AL-1  
10 antibodies are particularly useful in the detection of AL-1 in clinical samples for diagnostic purposes, and in the purification of AL-1.

AL-1, its derivatives, or its antibodies are formulated with physiologically acceptable carriers, especially for therapeutic use. Such carriers are used, for example, to provide sustained-release formulations of AL-1.

15        In further aspects, the invention provides a method for determining the presence of a nucleic acid molecule encoding AL-1 in test samples prepared from cells, tissues, or biological fluids, comprising contacting the test sample with isolated DNA comprising all or a portion of the nucleotide coding sequence for AL-1 and determining whether the isolated DNA hybridizes to a nucleic acid molecule in the test sample. DNA comprising all or a portion of the nucleotide coding sequence for AL-1 is also used in hybridization assays to  
20 identify and to isolate nucleic acids sharing substantial sequence identity to the coding sequence for AL-1, such as nucleic acids that encode allelic variants of AL-1.

Also provided is a method for amplifying a nucleic acid molecule encoding AL-1 that is present in a test sample, comprising the use of an oligonucleotide having a portion of the nucleotide coding sequence for AL-1 as a primer in a polymerase chain reaction.

#### 25        Brief Description of the Drawings

Figure 1 shows the nucleotide coding sequence (SEQ. ID. NO: 1), and the deduced amino acid sequence (SEQ. ID. NO: 2) encoded by the isolated REK7 cDNA. The N-terminus of the mature REK7 protein is indicated by a rightward arrow and the C-terminus of the REK7 extracellular domain is indicated by a vertical arrow.

30        Figure 2 shows the nucleotide coding sequence (SEQ. ID. NO: 3), and the deduced amino acid sequence (SEQ. ID. NO: 4) encoded by the isolated AL-1 cDNA. The N-terminus of the mature AL-1 protein is indicated by a rightward arrow (the mature protein begins with amino acid residue number 21). The underlined sequences corresponds to the sequences obtained by sequencing of purified BT20 cell-derived AL-1. The shaded boxes indicate potential N-glycosylation sites. The unshaded box shows the C-terminal hydrophobic  
35 domain and the upward arrow indicates a potential attachment site for glycosphosphatidyl-inositol (GPI). The anchor is likely to be at Asn-103. As taught herein AL-1 expressed on the surface of BT20 cells is GPI anchored.

Figure 3 shows a comparison of the AL-1 sequence with that of ELF-1 (Cheng and Flanagan, *Cell* 79:157-168 (1994)), B61 (Bartley et al., *Nature* 368:558-560 (1994)), EHK-1-L (Davis et al. *Science* 266:816-819 (1994)) and the extracellular domain of LERK2 (Beckmann et al., *EMBO J.* 13:3757-3762 (1994)). Identical amino acids are boxed, and gaps introduced for optimal alignment are indicated by dashes. Shaded areas highlight conserved cysteine residues.

#### Detailed Description of the Preferred Embodiments

"AL-1" or "AL-1 protein" refers to a polypeptide or protein encoded by the AL-1 nucleotide sequence set forth in Figure 2; a polypeptide that is the translated amino acid sequence set forth in Figure 2; fragments thereof having greater than about 5 amino acid residues and comprising an immune epitope or other biologically active site of AL-1; amino acid sequence variants of the amino acid sequence set forth in Figure 2 wherein one or more amino acid residues are added at the N- or C-terminus of, or within, said Figure 2 sequence or its fragments as defined above; amino acid sequence variants of said Figure 2 sequence or its fragments as defined above wherein one or more amino acid residues of said Figure 2 sequence or fragment thereof are deleted, and optionally substituted by one or more amino acid residues; and derivatives of the above proteins, polypeptides, or fragments thereof, wherein an amino acid residue has been covalently modified so that the resulting product is a non-naturally occurring amino acid. AL-1 amino acid sequence variants may be made synthetically, for example, by site-directed or PCR mutagenesis, or may exist naturally, as in the case of allelic forms and other naturally occurring variants of the translated amino acid sequence set forth in Figure 2 that may occur in human and other animal species. In any event, such fragments, variants, and derivatives exclude any polypeptide heretofore identified, including any known neurotrophic factor, such as nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5), as well as statutorily obvious variants thereof.

An AL-1 amino acid sequence variant is included within the scope of the invention provided that it is functionally active. As used herein, "functionally active" and "functional activity" in reference to AL-1 means that the AL-1 is able to promote or enhance the growth, survival, function, and/or differentiation of neurons and glia, especially axon fasciculation and process outgrowth, whether the neurons be central, peripheral, motoneurons, or sensory neurons, e.g. photoreceptors, vestibular ganglia, spinal ganglia, auditory hair cells, and/or that the AL-1 is immunologically cross-reactive with an antibody directed against an epitope of naturally occurring AL-1. Therefore, AL-1 amino acid sequence variants generally will share at least about 75% (preferably greater than 80% and more preferably greater than 90%) sequence identity with the translated amino acid sequence set forth in Figure 2, after aligning the sequences to provide for maximum homology, as determined, for example, by the Fitch, et al., *Proc. Nat. Acad. Sci. USA* 80:1382-1386 (1983), version of the algorithm described by Needleman, et al., *J. Mol. Biol.* 48:443-453 (1970).

Amino acid sequence variants of AL-1 are prepared by introducing appropriate nucleotide changes into AL-1 DNA and thereafter expressing the resulting modified DNA in a host cell, or by *in vitro* synthesis. Such variants include, for example, deletions from, or insertions or substitutions of, amino acid residues within the AL-1 amino acid sequence set forth in Figure 2. Any combination of deletion, insertion, and substitution may

be made to arrive at an amino acid sequence variant of AL-1, provided that such variant possesses the desired characteristics described herein. Changes that are made in the amino acid sequence set forth in Figure 2 to arrive at an amino acid sequence variant of AL-1 also may result in further modifications of AL-1 upon its expression in host cells, for example, by virtue of such changes introducing or moving sites of glycosylation, or introducing  
5 membrane anchor sequences as described, for example, in PCT Pat. Pub. No. WO 89/01041 (published February 9, 1989).

There are two principal variables in the construction of amino acid sequence variants of AL-1: the location of the mutation site and the nature of the mutation. These are variants from the amino acid sequence set forth in Figure 2, and may represent naturally occurring allelic forms of AL-1, or predetermined mutant  
10 forms of AL-1 made by mutating AL-1 DNA, either to arrive at an allele or a variant not found in nature. In general, the location and nature of the mutation chosen will depend upon the AL-1 characteristic to be modified.

For example, due to the degeneracy of nucleotide coding sequences, mutations can be made in the AL-1 nucleotide sequence set forth in Figure 2 without affecting the amino acid sequence of the AL-1 encoded thereby. Other mutations can be made that will result in a AL-1 that has an amino acid sequence different from  
15 that set forth in Figure 2, but which is functionally active. Such functionally active amino acid sequence variants of AL-1 are selected, for example, by substituting one or more amino acid residues in the amino acid sequence set forth in Figure 2 with other amino acid residues of a similar or different polarity or charge.

One useful approach is called "alanine scanning mutagenesis." Here, a an amino acid residue or group of target residues are identified (e.g., charged residues such as arg, asp, his, lys, and glu) and, by means of  
20 recombinant DNA technology, replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Cunningham, et al., Science 244: 1081-1085 (1989). Those domains demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at or for the sites of substitution. Obviously, such variations that, for example, convert the amino acid sequence set forth in Figure  
25 2 to the amino acid sequence of a known neurotrophic factor, such as NGF, BDNF, NT-3, NT-4/5, or another known polypeptide or protein (see Figure 3) are not included within the scope of this invention, nor are any other fragments, variants, and derivatives of the amino acid AL-1 that are not novel and unobvious over the prior art. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation per se need not be predetermined. For example, to optimize the performance of a mutation at a given  
30 site, ala scanning or random mutagenesis is conducted at the target codon or region and the expressed AL-1 variants are screened for functional activity.

Amino acid sequence deletions generally range from about 1 to 30 residues, more preferably about 1 to 10 residues, and typically are contiguous. Deletions from regions of substantial homology with other tyrosine kinase receptor ligands, for example, are more likely to affect the functional activity of AL-1. Generally, the  
35 number of consecutive deletions will be selected so as to preserve the tertiary structure of AL-1 in the affected domain, e.g., beta-pleated sheet or alpha helix.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one amino acid residue to polypeptides containing a hundred or more residues, as well as intrasequence



insertions of single or multiple amino acid residues. Intrasequence insertions (i.e., insertions made within the amino acid sequence set forth in Figure 2) may range generally from about 1 to 10 residues, more preferably 1 to 5, most preferably 1 to 3. Examples of terminal insertions include AL-1 with an N-terminal methionyl residue (such as may result from the direct expression of AL-1 in recombinant cell culture), and AL-1 with a  
5 heterogeneous N-terminal signal sequence to improve the secretion of AL-1 from recombinant host cells. Such signal sequences generally will be homologous to the host cell used for expression of AL-1, and include STII or lpp for *E. coli*, alpha factor for yeast, and viral signals such as herpes gD for mammalian cells. Other insertions include the fusion to the N- or C-terminus of AL-1 of immunogenic polypeptides (for example, bacterial polypeptides such as beta-lactamase or an enzyme encoded by the *E. coli* trp locus, or yeast protein),  
10 and C-terminal fusions with proteins having a long half-life such as immunoglobulin constant regions, albumin, or ferritin, as described in PCT Pat. Pub. No. WO 89/02922 (published April 6, 1989).

The third group of variants are those in which at least one amino acid residue in the amino acid sequence set forth in Figure 2, preferably one to four, more preferably one to three, even more preferably one to two, and most preferably only one, has been removed and a different residue inserted in its place. The sites  
15 of greatest interest for making such substitutions are in the regions of the amino acid sequence set forth in Figure 2 that have the greatest homology with other tyrosine kinase receptor ligands (for non-limiting examples, see Figure 3). Those sites are likely to be important to the functional activity of the AL-1. Accordingly, to retain functional activity, those sites, especially those falling within a sequence of at least three other identically conserved sites, are substituted in a relatively conservative manner. Such conservative substitutions are shown  
20 in Table 1 under the heading of preferred substitutions. If such substitutions do not result in a change in functional activity, then more substantial changes, denominated exemplary substitutions in Table 1, or as further described below in reference to amino acid classes, may be introduced and the resulting variant AL-1 analyzed for functional activity.

Table 1

	<u>Original Residue</u>	<u>Exemplary Substitutions</u>	<u>Preferred Substitutions</u>
	Ala (A)	val; leu; ile	val
5	Arg (R)	lys; gln; asn	lys
	Asn (N)	gln; his; lys; arg	gln
	Asp (D)	glu	glu
	Cys (C)	ser	ser
	Gln (Q)	asn	asn
10	Glu (E)	asp	asp
	Gly (G)	pro	pro
	His (H)	asn; gln; lys; arg	arg
	Ile (I)	leu; val; met; ala; phe; norleucine	leu
15	Leu (L)	norleucine; ile; val; met; ala; phe	ile
	Lys (K)	arg; gln; asn	arg
	Met (M)	leu; phe; ile	leu
	Phe (F)	leu; val; ile; ala	leu
20	Pro (P)	gly	gly
	Ser (S)	thr	thr
	Thr (T)	ser	ser
	Trp (W)	tyr	tyr
	Tyr (Y)	trp; phe; thr; ser	phe
25	Val (V)	ile; leu; met; phe; ala; norleucine	leu

Insertional, deletional, and substitutional changes in the amino acid sequence set forth in Figure 2 may be made to improve the stability of AL-1. For example, trypsin or other protease cleavage sites are identified by inspection of the encoded amino acid sequence for an arginyl or lysinyl residue. These are rendered inactive to protease by substituting the residue with another residue, preferably a basic residue such as glutamine or a hydrophobic residue such as serine; by deleting the residue; or by inserting a prolyl residue immediately after the residue. Also, any cysteine residues not involved in maintaining the proper conformation of AL-1 for functional activity may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking.

Figure 3 shows a comparison of AL-1 sequence with that of other sequences and indicates AL-1 is a new member of an emerging ligand family. ELF-1 is a ligand for the Eph-related receptors HEK and SEK, and is the most closely related to AL-1 (50.5% identity). B61 shows 442.9% similarity and EHKL-1, a reported ligand for EHK-1/REK7, shows 41.4% identity. Accordingly, additional sites for mutation are those sites that

are conserved in AL-1 (or alternatively REK7) amongst species variants of AL-1 (or REK7) but are not conserved between AL-1 and another ligand in the eph family, preferably between AL-1 and at least two ligands, and more preferably at least three ligands. Such sites are candidates sites for modulating receptor specificity and selectivity.

- 5 Covalent modifications of AL-1 molecules also are included within the scope of this invention. For example, covalent modifications are introduced into AL-1 by reacting targeted amino acid residues of the AL-1 with an organic derivatizing agent that is capable of reacting with selected amino acid side chains or the N- or C-terminal residues.

Cysteiny l residues most commonly are reacted with  $\alpha$ -haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteiny l residues also are derivatized by reaction with bromotrifluoroacetone,  $\alpha$ -bromo- $\beta$ -(5-imidazolyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylpyrocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1M sodium cacodylate at pH 6.0.

Lysiny l and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysiny l residues. Other suitable reagents for derivatizing  $\alpha$ -amino-containing residues include imidoesters such as methyl picolinimide; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4-pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginy l residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK<sub>a</sub> of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

The specific modification of tyrosyl residues may be made, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidazole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues are iodinated using <sup>125</sup>I or <sup>131</sup>I to prepare labeled proteins for use in radioimmunoassay, the chloramine T method described above being suitable.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R'-N=C=N-R'), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginy l and glutaminy l residues by reaction with ammonium ions.

Derivatization with bifunctional agents is useful for crosslinking AL-1 to a water-insoluble support matrix or surface for use in the method for purifying anti-AL-1 antibodies, or for therapeutic use. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxy-

succinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimide yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Pat. Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues, respectively. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the  $\alpha$ -amino groups of lysine, arginine, and histidine side chains, acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group. Creighton, Proteins: Structure and Molecular Properties, pp.79-86 (W.H. Freeman & Co., 1983). AL-1 also is covalently linked to nonproteinaceous polymers, e.g. polyethylene glycol, polypropylene glycol or polyoxyalkylenes, in the manner set forth in U.S. Pat. Nos. 4,179,337; 4,301,144; 4,496,689; 4,640,835; 4,670,417; or 4,791,192.

"AL-1 antagonist" or "antagonist" refers to a substance that opposes or interferes with a functional activity of AL-1.

"Cell," "host cell," "cell line," and "cell culture" are used interchangeably and all such terms should be understood to include progeny. Thus, the words "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of times the cultures have been passaged. It should also be understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations.

"Plasmids" are DNA molecules that are capable of replicating within a host cell, either extrachromosomally or as part of the host cell chromosome(s), and are designated by a lower case "p" preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are commercially available, are publicly available on an unrestricted basis, or can be constructed from such available plasmids as disclosed herein and/or in accordance with published procedures. In certain instances, as will be apparent to the ordinarily skilled artisan, other plasmids known in the art may be used interchangeably with plasmids described herein.

"Control sequences" refers to DNA sequences necessary for the expression of an operably linked nucleotide coding sequence in a particular host cell. The control sequences that are suitable for expression in prokaryotes, for example, include origins of replication, promoters, ribosome binding sites, and transcription termination sites. The control sequences that are suitable for expression in eukaryotes, for example, include origins of replication, promoters, ribosome binding sites, polyadenylation signals, and enhancers.

An "exogenous" element is one that is foreign to the host cell, or homologous to the host cell but in a position within the host cell in which the element is ordinarily not found.

"Digestion" of DNA refers to the catalytic cleavage of DNA with an enzyme that acts only at certain locations in the DNA. Such enzymes are called restriction enzymes or restriction endonucleases, and the sites

within DNA where such enzymes cleave are called restriction sites. If there are multiple restriction sites within the DNA, digestion will produce two or more linearized DNA fragments (restriction fragments). The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors, and other requirements as established by the enzyme manufacturers are used. Restriction enzymes commonly are designated by abbreviations composed of a capital letter followed by other letters representing the micro-organism from which each restriction enzyme originally was obtained and then a number designating the particular enzyme. In general, about 1 µg of DNA is digested with about 1-2 units of enzyme in about 20 µl of buffer solution. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer, and/or are well known in the art.

"Recovery" or "isolation" of a given fragment of DNA from a restriction digest typically is accomplished by separating the digestion products, which are referred to as "restriction fragments," on a polyacrylamide or agarose gel by electrophoresis, identifying the fragment of interest on the basis of its mobility relative to that of marker DNA fragments of known molecular weight, excising the portion of the gel that contains the desired fragment, and separating the DNA from the gel, for example by electroelution.

"Ligation" refers to the process of forming phosphodiester bonds between two double-stranded DNA fragments. Unless otherwise specified, ligation is accomplished using known buffers and conditions with 10 units of T4 DNA ligase per 0.5 µg of approximately equimolar amounts of the DNA fragments to be ligated.

"Oligonucleotides" are short-length, single- or double-stranded polydeoxynucleotides that are chemically synthesized by known methods (involving, for example, triester, phosphoramidite, or phosphonate chemistry), such as described by Engels, et al., *Agnew. Chem. Int. Ed. Engl.* 28:716-734 (1989). They are then purified, for example, by polyacrylamide gel electrophoresis.

"Polymerase chain reaction," or "PCR," as used herein generally refers to a method for amplification of a desired nucleotide sequence *in vitro*, as described in U.S. Pat. No. 4,683,195. In general, the PCR method involves repeated cycles of primer extension synthesis, using two oligonucleotide primers capable of hybridizing preferentially to a template nucleic acid. Typically, the primers used in the PCR method will be complementary to nucleotide sequences within the template at both ends of or flanking the nucleotide sequence to be amplified, although primers complementary to the nucleotide sequence to be amplified also may be used. Wang, et al., in PCR Protocols, pp.70-75 (Academic Press, 1990); Ochman, et al., in PCR Protocols, pp. 219-227; Triglia, et al., *Nuc. Acids Res.* 16:8186 (1988).

"PCR cloning" refers to the use of the PCR method to amplify a specific desired nucleotide sequence that is present amongst the nucleic acids from a suitable cell or tissue source, including total genomic DNA and cDNA transcribed from total cellular RNA. Frohman, et al., *Proc. Nat. Acad. Sci. USA* 85:8998-9002 (1988); Saiki, et al., *Science* 239:487-492 (1988); Mullis, et al., *Meth. Enzymol.* 155:335-350 (1987).

"Stringent conditions" for hybridization or annealing of nucleic acid molecules are those that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate (SDS) at 50°C, or (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at

42°C. Another example is use of 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 µg/mL), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC and 0.1% SDS.

"AL-1 nucleic acid" is RNA or DNA that encodes AL-1. "AL-1 DNA" is DNA that encodes AL-1.

5 AL-1 DNA is obtained from cDNA or genomic DNA libraries, or by *in vitro* synthesis. Identification of AL-1 DNA within a cDNA or a genomic DNA library, or in some other mixture of various DNAs, is conveniently accomplished by the use of an oligonucleotide hybridization probe that is labeled with a detectable moiety, such as a radioisotope. Keller, et al., *DNA Probes*, pp.149-213 (Stockton Press, 1989). To identify DNA encoding AL-1, the nucleotide sequence of the hybridization probe preferably is selected so that the hybridization probe  
10 is capable of hybridizing preferentially to DNA encoding the AL-1 amino acid sequence set forth in Figure 2, or a variant or derivative thereof as described herein, under the hybridization conditions chosen. Another method for obtaining AL-1 nucleic acid is to chemically synthesize it using one of the methods described, for example, by Engels, et al., *Agnew. Chem. Int. Ed. Engl.* 28:716-734 (1989).

If the entire nucleotide coding sequence for AL-1 is not obtained in a single cDNA, genomic DNA,  
15 or other DNA, as determined, for example, by DNA sequencing or restriction endonuclease analysis, then appropriate DNA fragments (e.g., restriction fragments or PCR amplification products) may be recovered from several DNAs and covalently joined to one another to construct the entire coding sequence. The preferred means of covalently joining DNA fragments is by ligation using a DNA ligase enzyme, such as T4 DNA ligase.

"Isolated" AL-1 nucleic acid is AL-1 nucleic acid that is identified and separated from (or otherwise  
20 substantially free from), contaminant nucleic acid encoding other polypeptides. The isolated AL-1 nucleic acid can be incorporated into a plasmid or expression vector, or can be labeled for diagnostic and probe purposes, using a label as described further herein in the discussion of diagnostic assays and nucleic acid hybridization methods.

For example, isolated AL-1 DNA, or a fragment thereof comprising at least about 15 nucleotides, is  
25 used as a hybridization probe to detect, diagnose, or monitor disorders or diseases that involve changes in AL-1 expression, such as may result from neuron damage. In one embodiment of the invention, total RNA in a tissue sample from a patient (that is, a human or other mammal) can be assayed for the presence of AL-1 messenger RNA, wherein the decrease in the amount of AL-1 messenger RNA is indicative of neuronal degeneration.

Isolated AL-1 nucleic acid also is used to produce AL-1 by recombinant DNA and recombinant cell  
30 culture methods. In various embodiments of the invention, host cells are transformed or transfected with recombinant DNA molecules comprising an isolated AL-1 DNA, to obtain expression of the AL-1 DNA and thus the production of AL-1 in large quantities. DNA encoding amino acid sequence variants of AL-1 is prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants of AL-1) or preparation by site-  
35 directed (or oligonucleotide-mediated) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared DNA encoding a variant or a non-variant form of AL-1.

Site-directed mutagenesis is a preferred method for preparing substitution, deletion, and insertion variants of AL-1 DNA. This technique is well known in the art, Zoller, et al., *Meih. Erz.* 100:4668-500 (1983);

Zoller, et al., *Meth. Enz.* 154:329-350 (1987); Carter, *Meth. Enz.* 154:382-403 (1987); Horwitz, et al., *Meth. Enz.* 185:599-611 (1990), and has been used, for example, to produce amino acid sequence variants of trypsin and T4 lysozyme, which variants have certain desired functional properties. Perry, et al., *Science* 226:555-557 (1984); Craik, et al., *Science* 228:291-297 (1985).

5 Briefly, in carrying out site-directed mutagenesis of AL-1 DNA, the AL-1 DNA is altered by first hybridizing an oligonucleotide encoding the desired mutation to a single strand of such AL-1 DNA. After hybridization, a DNA polymerase is used to synthesize an entire second strand, using the hybridized oligonucleotide as a primer, and using the single strand of AL-1 DNA as a template. Thus, the oligonucleotide encoding the desired mutation is incorporated in the resulting double-stranded DNA.

10 Oligonucleotides for use as hybridization probes or primers may be prepared by any suitable method, such as by purification of a naturally occurring DNA or by *in vitro* synthesis. For example, oligonucleotides are readily synthesized using various techniques in organic chemistry, such as described by Narang, et al., *Meth. Enzymol.* 68:90-98 (1979); Brown, et al., *Meth. Enzymol.* 68:109-151 (1979); Caruther, et al., *Meth. Enzymol.* 154:287-313 (1985). The general approach to selecting a suitable hybridization probe or primer is well known.

15 Keller, et al., DNA Probes, pp.11-18 (Stockton Press, 1989). Typically, the hybridization probe or primer will contain 10-25 or more nucleotides, and will include at least 5 nucleotides on either side of the sequence encoding the desired mutation so as to ensure that the oligonucleotide will hybridize preferentially to the single-stranded DNA template molecule.

Multiple mutations are introduced into AL-1 DNA to produce amino acid sequence variants of AL-1

20 comprising several or a combination of insertions, deletions, or substitutions of amino acid residues as compared to the amino acid sequence set forth in Figure 2. If the sites to be mutated are located close together, the mutations may be introduced simultaneously using a single oligonucleotide that encodes all of the desired mutations. If, however, the sites to be mutated are located some distance from each other (separated by more than about ten nucleotides), it is more difficult to generate a single oligonucleotide that encodes all of the desired

25 changes. Instead, one of two alternative methods may be employed.

In the first method, a separate oligonucleotide is generated for each desired mutation. The oligonucleotides are then annealed to the single-stranded template DNA simultaneously, and the second strand of DNA that is synthesized from the template will encode all of the desired amino acid substitutions.

The alternative method involves two or more rounds of mutagenesis to produce the desired mutant.

30 The first round is as described for introducing a single mutation: a single strand of a previously prepared AL-1 DNA is used as a template, an oligonucleotide encoding the first desired mutation is annealed to this template, and a heteroduplex DNA molecule is then generated. The second round of mutagenesis utilizes the mutated DNA produced in the first round of mutagenesis as the template. Thus, this template already contains one or more mutations. The oligonucleotide encoding the additional desired amino acid substitution(s) is then annealed

35 to this template, and the resulting strand of DNA now encodes mutations from both the first and second rounds of mutagenesis. This resultant DNA can be used as a template in a third round of mutagenesis, and so on.

PCR mutagenesis is also suitable for making amino acid sequence variants of AL-1. Higuchi, in PCR Protocols, pp.177-183 (Academic Press, 1990); Vallette, et al., *Nuc. Acids Res.* 17:723-733 (1989). Briefly,

when small amounts of template DNA are used as starting material in a PCR, primers that differ slightly in sequence from the corresponding region in a template DNA can be used to generate relatively large quantities of a specific DNA fragment that differs from the template sequence only at the positions where the primers differ from the template. For introduction of a mutation into a plasmid DNA, for example, one of the primers is designed to overlap the position of the mutation and to contain the mutation; the sequence of the other primer must be identical to a nucleotide sequence within the opposite strand of the plasmid DNA, but this sequence can be located anywhere along the plasmid DNA. It is preferred, however, that the sequence of the second primer is located within 200 nucleotides from that of the first, such that in the end the entire amplified region of DNA bounded by the primers can be easily sequenced. PCR amplification using a primer pair like the one just described results in a population of DNA fragments that differ at the position of the mutation specified by the primer, and possibly at other positions, as template copying is somewhat error-prone. Wagner, et al., in PCR Topics, pp.69-71 (Springer-Verlag, 1991).

If the ratio of template to product amplified DNA is extremely low, the majority of product DNA fragments incorporate the desired mutation(s). This product DNA is used to replace the corresponding region in the plasmid that served as PCR template using standard recombinant DNA methods. Mutations at separate positions can be introduced simultaneously by either using a mutant second primer, or performing a second PCR with different mutant primers and ligating the two resulting PCR fragments simultaneously to the plasmid fragment in a three (or more)-part ligation.

Another method for preparing variants, cassette mutagenesis, is based on the technique described by Wells et al., *Gene*, 34:315-323 (1985). The starting material is the plasmid (or other vector) comprising the AL-1 DNA to be mutated. The codon(s) in the AL-1 DNA to be mutated are identified. There must be a unique restriction endonuclease site on each side of the identified mutation site(s). If no such restriction sites exist, they may be generated using the above-described oligonucleotide-mediated mutagenesis method to introduce them at appropriate locations in the AL-1 DNA. The plasmid DNA is cut at these sites to linearize it. A double-stranded oligonucleotide encoding the sequence of the DNA between the restriction sites but containing the desired mutation(s) is synthesized using standard procedures, wherein the two strands of the oligonucleotide are synthesized separately and then hybridized together using standard techniques. This double-stranded oligonucleotide is referred to as the cassette. This cassette is designed to have 5' and 3' ends that are compatible with the ends of the linearized plasmid, such that it can be directly ligated to the plasmid. This plasmid now contains the mutated AL-1 DNA sequence.

AL-1 DNA, whether cDNA or genomic DNA or a product of *in vitro* synthesis, is ligated into a replicable vector for further cloning or for expression. "Vectors" are plasmids and other DNAs that are capable of replicating autonomously within a host cell, and as such, are useful for performing two functions in conjunction with compatible host cells (a vector-host system). One function is to facilitate the cloning of the nucleic acid that encodes the AL-1, i.e., to produce usable quantities of the nucleic acid. The other function is to direct the expression of AL-1. One or both of these functions are performed by the vector-host system. The vectors will contain different components depending upon the function they are to perform as well as the host cell with which they are to be used for cloning or expression.



To produce AL-1, an expression vector will contain nucleic acid that encodes AL-1 as described above. The AL-1 of this invention is expressed directly in recombinant cell culture, or as a fusion with a heterologous polypeptide, preferably a signal sequence or other polypeptide having a specific cleavage site at the junction between the heterologous polypeptide and the AL-1.

5 In one example of recombinant host cell expression, mammalian cells are transfected with an expression vector comprising AL-1 DNA and the AL-1 encoded thereby is recovered from the culture medium in which the recombinant host cells are grown. But the expression vectors and methods disclosed herein are suitable for use over a wide range of prokaryotic and eukaryotic organisms.

Prokaryotes may be used for the initial cloning of DNAs and the construction of the vectors useful in  
10 the invention. However, prokaryotes may also be used for expression of DNA encoding AL-1. Polypeptides that are produced in prokaryotic host cells typically will be non-glycosylated.

Plasmid or viral vectors containing replication origins and other control sequences that are derived from species compatible with the host cell are used in connection with prokaryotic host cells, for cloning or expression of an isolated DNA. For example, *E. coli* typically is transformed using pBR322, a plasmid derived  
15 from an *E. coli* species. Bolivar, et al., *Gene* 2:95-113 (1987). pBR322 contains genes for ampicillin and tetracycline resistance so that cells transformed by the plasmid can easily be identified or selected. For it to serve as an expression vector, the pBR322 plasmid, or other plasmid or viral vector, must also contain, or be modified to contain, a promoter that functions in the host cell to provide messenger RNA (mRNA) transcripts of a DNA inserted downstream of the promoter. Rangagwala, et al., *BioTechnology* 9:477-479 (1991).

20 In addition to prokaryotes, eukaryotic microbes, such as yeast, may also be used as hosts for the cloning or expression of DNAs useful in the invention. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used eukaryotic microorganism. Plasmids useful for cloning or expression in yeast cells of a desired DNA are well known, as are various promoters that function in yeast cells to produce mRNA transcripts.

Furthermore, cells derived from multicellular organisms also may be used as hosts for the cloning or  
25 expression of DNAs useful in the invention. Mammalian cells are most commonly used, and the procedures for maintaining or propagating such cells *in vitro*, which procedures are commonly referred to as tissue culture, are well known. Kruse & Patterson, eds., Tissue Culture (Academic Press, 1977). Examples of useful mammalian cells are human cell lines such as 293, HeLa, and WI-38, monkey cell lines such as COS-7 and VERO, and hamster cell lines such as BHK-21 and CHO, all of which are publicly available from the American  
30 Type Culture Collection (ATCC), Rockville, Maryland 20852 USA.

Expression vectors, unlike cloning vectors, should contain a promoter that is recognized by the host organism and is operably linked to the AL-1 nucleic acid. Promoters are untranslated sequences that are located upstream from the start codon of a gene and that control transcription of the gene (that is, the synthesis of mRNA). Promoters typically fall into two classes, inducible and constitutive. Inducible promoters are  
35 promoters that initiate high level transcription of the DNA under their control in response to some change in culture conditions, for example, the presence or absence of a nutrient or a change in temperature.

A large number of promoters are known, that may be operably linked to AL-1 DNA to achieve expression of AL-1 in a host cell. This is not to say that the promoter associated with naturally occurring AL-1

DNA is not usable. However, heterologous promoters generally will result in greater transcription and higher yields of expressed AL-1.

Promoters suitable for use with prokaryotic hosts include the  $\beta$ -lactamase and lactose promoters. Goeddel, et al., *Nature* 281:544-548 (1979), tryptophan (trp) promoter, Goeddel, et al., *Nuc. Acids Res.* 8:4057-4074 (1980), and hybrid promoters such as the tac promoter, deBoer, et al., *Proc. Natl. Acad. Sci. USA* 80:21-25 (1983). However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, Siebenlist, et al., *Cell* 20:269-281 (1980), thereby enabling a skilled worker operably to ligate them to DNA encoding AL-1 using linkers or adaptors to supply any required restriction sites. Wu, et al., *Meth. Enz.* 152:343-349 (1987).

Suitable promoters for use with yeast hosts include the promoters for 3-phosphoglycerate kinase, Hitzeman, et al., *J. Biol. Chem.* 255:12073-12080 (1980); Kingsman, et al., *Meth. Enz.* 185:329-341 (1990), or other glycolytic enzymes such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Dodson, et al., *Nuc. Acids res.* 10:2625-2637 (1982); Emr, *Meth. Enz.* 185:231-279 (1990).

Expression vectors useful in mammalian cells typically include a promoter derived from a virus. For example, promoters derived from polyoma virus, adenovirus, cytomegalovirus (CMV), and simian virus 40 (SV40) are commonly used. Further, it is also possible, and often desirable, to utilize promoter or other control sequences associated with a naturally occurring DNA that encodes AL-1, provided that such control sequences are functional in the particular host cell used for recombinant DNA expression.

Other control sequences that are desirable in an expression vector in addition to a promoter are a ribosome binding site, and in the case of an expression vector used with eukaryotic host cells, an enhancer. Enhancers are cis-acting elements of DNA, usually about from 10-300 bp, that act on a promoter to increase the level of transcription. Many enhancer sequences are now known from mammalian genes (for example, the genes for globin, elastase, albumin,  $\alpha$ -fetoprotein and insulin). Typically, however, the enhancer used will be one from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. Kriegler, *Meth. Enz.* 185:512-527 (1990).

Expression vectors may also contain sequences necessary for the termination of transcription and for stabilizing the messenger RNA (mRNA). Balbas, et al., *Meth. Enz.* 185:14-37 (1990); Levinson, *Meth. Enz.* 185:485-511 (1990). In the case of expression vectors used with eukaryotic host cells, such transcription termination sequences may be obtained from the untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain polyadenylation sites as well as transcription termination sites. Birnstein, et al., *Cell* 41:349-359 (1985).

In general, control sequences are DNA sequences necessary for the expression of an operably linked coding sequence in a particular host cell. "Expression" refers to transcription and/or translation. "Operably linked" refers to the covalent joining of two or more DNA sequences, by means of enzymatic ligation or otherwise, in a configuration relative to one another such that the normal function of the sequences can be

performed. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used, in conjunction with standard recombinant DNA methods.

Expression and cloning vectors also will contain a sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosome(s), and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most gram-negative bacteria, the 2 $\mu$  plasmid origin is suitable for yeast, and various viral origins (for example, from SV40, polyoma, or adenovirus) are useful for cloning vectors in mammalian cells. Most expression vectors are "shuttle" vectors, i.e. they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector may be cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

The expression vector may also include an amplifiable gene, such as that comprising the coding sequence for dihydrofolate reductase (DHFR). Cells containing an expression vector that includes a DHFR gene may be cultured in the presence of methotrexate, a competitive antagonist of DHFR. This leads to the synthesis of multiple copies of the DHFR gene and, concomitantly, multiple copies of other DNA sequences comprising the expression vector, Ringold, et al., *J. Mol. Appl. Genet.* 1:165-175 (1981), such as a DNA sequence encoding AL-1. In that manner, the level of AL-1 produced by the cells may be increased.

DHFR protein encoded by the expression vector also may be used as a selectable marker of successful transfection. For example, if the host cell prior to transformation is lacking in DHFR activity, successful transformation by an expression vector comprising DNA sequences encoding AL-1 and DHFR protein can be determined by cell growth in medium containing methotrexate. Also, mammalian cells transformed by an expression vector comprising DNA sequences encoding AL-1, DHFR protein, and aminoglycoside 3' phosphotransferase (APH) can be determined by cell growth in medium containing an aminoglycoside antibiotic such as kanamycin or neomycin. Because eukaryotic cells do not normally express an endogenous APH activity, genes encoding APH protein, commonly referred to as neo' genes, may be used as dominant selectable markers in a wide range of eukaryotic host cells, by which cells transfected by the vector can easily be identified or selected. Jiminez, et al., *Nature*, 287:869-871 (1980); Colbere-Garapin, et al., *J. Mol. Biol.* 150:1-14 (1981); Okayama & Berg, *Mol. Cell. Biol.*, 3:280-289 (1983).

Many other selectable markers are known that may be used for identifying and isolating recombinant host cells that express AL-1. For example, a suitable selection marker for use in yeast is the *trp1* gene present in the yeast plasmid YRp7. Stinchcomb, et al., *Nature* 282:39-43 (1979); Kingsman, et al., *Gene* 7:141-152

(1979); Tschemper, et al., *Gene* 10:157-166 (1980). The *trp1* gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 (available from the American Type Culture Collection, Rockville, Maryland 20852 USA). Jones, *Genetics* 85:12 (1977). The presence of the *trp1* lesion in the yeast host cell genome then provides an effective environment for  
5 detecting transformation by growth in the absence of tryptophan. Similarly, *Leu2*-deficient yeast strains (ATCC Nos. 20622 or 38626) are complemented by known plasmids bearing the *Leu2* gene.

Particularly useful in the invention are expression vectors that provide for the transient expression in mammalian cells of DNA encoding AL-1. In general, transient expression involves the use of an expression vector that is able to efficiently replicate in a host cell, such that the host cell accumulates many copies of the  
10 expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Yang, et al., *Cell* 47:3-10 (1986); Wong, et al., *Science* 228:810-815 (1985); Lee, et al., *Proc. Nat Acad. Sci. USA* 82:4360-4364 (1985). Thus, transient  
15 expression systems are particularly useful in the invention for expressing DNAs encoding amino acid sequence variants of AL-1, to identify those variants which are functionally active.

Since it is often difficult to predict in advance the characteristics of an amino acid sequence variant of AL-1, it will be appreciated that some screening of such variants will be needed to identify those that are functionally active. Such screening may be performed *in vitro*, using routine assays for receptor binding, or  
20 assays for axonal growth or development, or using immunoassays with monoclonal antibodies that selectively bind to AL-1 that is functionally active AL-1, such as a monoclonal antibody that selectively binds to the active site or receptor binding site of AL-1.

As used herein, the terms "transformation" and "transfection" refer to the process of introducing a desired nucleic acid, such a plasmid or an expression vector, into a host cell. Various methods of transformation  
25 and transfection are available, depending on the nature of the host cell. In the case of *E. coli* cells, the most common methods involve treating the cells with aqueous solutions of calcium chloride and other salts. In the case of mammalian cells, the most common methods are transfection mediated by either calcium phosphate or DEAE-dextran, or electroporation. Sambrook, et al., eds., Molecular Cloning, pp. 1.74-1.84 and 16.30-16.55 (Cold Spring Harbor Laboratory Press, 1989). Following transformation or transfection, the desired nucleic acid  
30 may integrate into the host cell genome, or may exist as an extrachromosomal element.

Host cells that are transformed or transfected with the above-described plasmids and expression vectors are cultured in conventional nutrient media modified as is appropriate for inducing promoters or selecting for drug resistance or some other selectable marker or phenotype. The culture conditions, such as temperature, pH,  
and the like, suitably are those previously used for culturing the host cell used for cloning or expression, as the  
35 case may be, and will be apparent those skilled in the art.

Suitable host cells for cloning or expressing the vectors herein are prokaryotes, yeasts, and higher eukaryotes, including insect, vertebrate, and mammalian host cells. Suitable prokaryotes include eubacteria,

such as Gram-negative or Gram-positive organisms, for example, E. coli, Bacillus species such as B. subtilis, Pseudomonas species such as P. aeruginosa, Salmonella typhimurium, or Serratia marcescans.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable hosts for AL-1-encoding vectors. Saccharomyces cerevisiae, or common baker's yeast, is the most commonly used  
5 among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as Schizosaccharomyces pombe, Beach and Nurse, *Nature* 290:140-142 (1981), Pichia pastoris, Cregg, et al., *Bio/Technology* 5:479-485 (1987); Sreekrishna, et al., *Biochemistry* 28:4117-4125 (1989), Neurospora crassa, Case, et al., *Proc. Natl. Acad. Sci. USA* 76:5259-5263 (1979), and Aspergillus hosts such as A. nidulans, Ballance, et al., *Biochem. Biophys. Res. Commun.* 112:284-289 (1983);  
10 Tilburn, et al., *Gene* 26:205-221 (1983); Yelton, et al., *Proc. Natl. Acad. Sci. USA* 81:1470-1474 (1984), and A. niger, Kelly, et al., *EMBO J.* 4:475-479 (1985).

Suitable host cells for the expression of AL-1 also are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is useable, whether from vertebrate or invertebrate culture. It will be appreciated, however, that because  
15 of the species-, tissue-, and cell-specificity of glycosylation, Rademacher, et al., *Ann. Rev. Biochem.* 57:785-838 (1988), the extent or pattern of glycosylation of AL-1 in a foreign host cell typically will differ from that of AL-1 obtained from a cell in which it is naturally expressed.

Examples of invertebrate cells include insect and plant cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as Spodoptera frugiperda (caterpillar), Aedes aegypti (mosquito), Aedes albopictus (mosquito), Drosophila melanogaster (fruitfly), and Bombyx mori host  
20 cells have been identified. Luckow, et al., *Bio/Technology* 6:47-55 (1988); Miller, et al., in *Genetic Engineering*, vol. 8, pp.277-279 (Plenum Publishing, 1986); Maeda, et al., *Nature* 315:592-594 (1985).

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically, plant cells are transfected by incubation with certain strains of the bacterium Agrobacterium tumefaciens, which has been previously altered to contain AL-1 DNA. During incubation of the plant cells with  
25 A. tumefaciens, the DNA encoding the AL-1 is transferred into cells, such that they become transfected, and will, under appropriate conditions, express the AL-1 DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences, and the ribulose biphosphate carboxylase promoter. Depicker, et al., *J. Mol. Appl. Gen.* 1:561-573  
30 (1982). Herrera-Estrella, et al., *Nature* 310:115-120 (1984). In addition, DNA segments isolated from the upstream region of the T-DNA 780 gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue. European Pat. Pub. No. EP 321,196 (published June 21, 1989).

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture  
35 (tissue culture) has become a routine procedure in recent years. Kruse & Patterson, eds., *Tissue Culture* (Academic Press, 1973). Examples of useful mammalian host cells are the monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line 293 (or 293 cells subcloned for growth in suspension culture), Graham, et al., *J. Gen Virol.* 36:59-72 (1977); baby hamster kidney cells

(BHK, ATCC CCL 10); Chinese hamster ovary cells (including DHFR-deficient CHO cells, Urlaub, et al., *Proc. Natl. Acad. Sci. USA* 77:4216-4220 (1980); mouse sertoli cells (TM4, Mather, *Biol. Reprod.* 23:243-251 (1980); monkey kidney cells (CV1, ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather, et al., *Annals N.Y. Acad. Sci.* 383:44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Construction of suitable vectors containing the nucleotide sequence encoding AL-1 and appropriate control sequences employs standard recombinant DNA methods. DNA is cleaved into fragments, tailored, and ligated together in the form desired to generate the vectors required.

For analysis to confirm correct sequences in the vectors constructed, the vectors are analyzed by restriction digestion (to confirm the presence in the vector of predicted restriction endonuclease) and/or by sequencing by the dideoxy chain termination method of Sanger, et al., *Proc. Nat. Acad. Sci. USA* 72:3918-3921 (1979).

The mammalian host cells used to produce the AL-1 of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium (MEM, Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium (DMEM, Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham, et al., *Meth. Enz.* 58:44-93 (1979); Barnes, et al., *Anal. Biochem.* 102:255-270 (1980); Bottenstein, et al., *Meth. Enz.* 58:94-109 (1979); U.S. Pat. Nos. 4,560,655; 4,657,866; 4,767,704; or 4,927,762; or in PCT Pat. Pub. Nos. WO 90/03430 (published April 5, 1990), may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics, trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The host cells referred to in this disclosure encompass cells in culture *in vitro* as well as cells that are within a host animal, for example, as a result of transplantation or implantation.

It is further contemplated that the AL-1 of this invention may be produced by homologous recombination, for example, as described in PCT Pat. Pub. No. WO 91/06667 (published May 16, 1991). Briefly, this method involves transforming cells containing an endogenous gene encoding AL-1 with a homologous DNA, which homologous DNA comprises (1) an amplifiable gene, such as DHFR, and (2) at least one flanking sequence, having a length of at least about 150 base pairs, which is homologous with a nucleotide sequence in the cell genome that is within or in proximity to the gene encoding AL-1. The transformation is carried out under conditions such that the homologous DNA integrates into the cell genome by recombination.

Cells having integrated the homologous DNA then are subjected to conditions which select for amplification of the amplifiable gene, whereby the AL-1 gene amplified concomitantly. The resulting cells then are screened for production of desired amounts of AL-1. Flanking sequences that are in proximity to a gene encoding AL-1 are readily identified, for example, by the method of genomic walking, using as a starting point the AL-1 nucleotide sequence set forth in Figure 2. Spoerel, et al., *Meth. Enz.* 152:598-603 (1987).

Gene amplification and/or gene expression may be measured in a sample directly, for example, by conventional Southern blotting to quantitate DNA, or Northern blotting to quantitate mRNA, using an appropriately labeled oligonucleotide hybridization probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, particularly  $^{32}\text{P}$ . However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radioisotopes, fluorophores, chromophores, or the like. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of the gene product, AL-1. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, luminescent labels, and the like. A particularly sensitive staining technique suitable for use in the present invention is described by Hsu, et al., *Am. J. Clin. Path.*, 75:734-738 (1980). Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal. Conveniently, the antibodies may be prepared against a synthetic peptide based on the DNA sequences provided herein.

AL-1 preferably is recovered from the culture medium as a secreted polypeptide, although it also may be recovered from host cell lysates. To obtain AL-1 that is substantially free of contaminating proteins or polypeptides of the host cell in which it is produced it is necessary to purify the AL-1, based on the differential physical properties of AL-1 as compared to the contaminants with which it may be associated. For example, as a first step, the culture medium or lysate is centrifuged to remove particulate cell debris. AL-1 thereafter is purified from contaminant soluble proteins and polypeptides, for example, by ammonium sulfate or ethanol precipitation, gel filtration (molecular exclusion chromatography), ion-exchange chromatography, immunoaffinity chromatography, reverse phase HPLC, and/or gel electrophoresis. For example, AL-1 can be purified by immunoaffinity chromatography using a REK7-IgG resin (comprising REK7-IgG coupled to the resin material), as described in Example 5. Secreted, soluble AL-1 resulting from removal of the GPI membrane anchor acts as an antagonist since it binds but does not activate its receptor as demonstrated in the Examples. In contrast, secreted, soluble AL-1-IgG in which AL-1 extracellular domain is

fused to an IgG Fc region acts as an AL-1 agonist. Its dimeric form may be responsible for its activity.

Amino acid sequence variants and derivatives of AL-1 are recovered in the same fashion, taking account of any distinguishing features or physical properties of the particular AL-1. For example, in the case of a fusion protein comprising AL-1 and another protein or polypeptide, such as a bacterial or viral antigen, a significant degree of purification may be obtained by using an immunoaffinity column containing antibody to the antigen. In any event, the ordinarily skilled artisan will appreciate that purification methods suitable for naturally occurring AL-1 may require modification to account for changes in the character of AL-1 or its variants or derivatives produced in recombinant host cells.

The purity of AL-1 produced according to the present invention is determined according to methods well known in the art, such as by analytical sodium dodecyl sulfate (SDS) gel electrophoresis, immunoassay, or amino acid composition or sequence analysis electrophoresis. Preferably, the AL-1 is purified to such an extent that it is substantially free of other proteins. For therapeutic uses, the purified AL-1 will be greater than 99% AL-1 and, accordingly, non-AL-1 proteins will comprise less than 1% of the total protein in the purified AL-1 composition.

AL-1 may be used as an immunogen to generate anti-AL-1 antibodies. Such antibodies, which specifically bind to AL-1, are useful as standards in assays for AL-1, such as by labeling purified AL-1 for use as a standard in a radioimmunoassay, enzyme-linked immunoassay, or competitive-type receptor binding assays radioreceptor assay, as well as in affinity purification techniques. Ordinarily, the anti-AL-1 antibody will bind AL-1 with an affinity of at least about  $10^6$  L/mole, and preferably at least about  $10^7$  L/mole.

Polyclonal antibodies directed toward AL-1 generally are raised in animals by multiple subcutaneous or intraperitoneal injections of AL-1 and an adjuvant. It may be useful to conjugate AL-1 or a peptide fragment thereof to a carrier protein that is immunogenic in the species to be immunized, such as keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor, using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (conjugation through lysine residues), glutaraldehyde, succinic anhydride,  $\text{SOCl}_2$ , or  $\text{R}^1\text{N} = \text{C} = \text{NR}$ , where R and  $\text{R}^1$  are different alkyl groups.

Animals are immunized with such AL-1-carrier protein conjugates combining 1 mg or 1  $\mu\text{g}$  of conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5th to 1/10th the original amount of conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. 7 to 14 days later animals are bled and the serum is assayed for anti-AL-1 antibody titer. Animals are boosted until the antibody titer plateaus. Preferably, the animal is boosted by injection with a conjugate of the same AL-1 with a different carrier protein and/or through a different cross-linking agent. Conjugates of AL-1 and a suitable carrier protein also can be made in recombinant cell culture as fusion proteins. Also, aggregating agents such as alum are used to enhance the immune response.

Monoclonal antibodies directed toward AL-1 are produced using any method which provides for the production of antibody molecules by continuous cell lines in culture. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is



not to be construed as requiring production of the antibody by any particular method. Examples of suitable methods for preparing monoclonal antibodies include the original hybridoma method of Kohler, et al., *Nature* 256:495-497 (1975), and the human B-cell hybridoma method, Kozbor, *J. Immunol.* 133:3001 (1984); Brodeur, et al., Monoclonal Antibody Production Techniques and Applications, pp.51-63 (Marcel Dekker, Inc., New York, 1987).

The monoclonal antibodies of the invention specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (Cabilly, et al., U.S. Patent No. 4,816,567; Morrison, et al., *Proc. Natl. Acad. Sci.* 81:6851-6855 (1984)).

In a preferred embodiment, the chimeric anti-AL-1 antibody is a "humanized" antibody. Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain.

Humanization can be performed following methods known in the art (Jones, et al., *Nature* 321:522-525 (1986); Riechmann, et al., *Nature*, 332:323-327 (1988); Verhoeven, et al., *Science* 239:1534-1536 (1988)), by substituting rodent complementarity-determining regions (CDRs) for the corresponding regions of a human antibody. Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region ( $J_H$ ) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, for example, Jakobovits, et al., *Proc. Natl. Acad. Sci.* 90: 2551-2555 (1993); Jakobovits, et al., *Nature* 362:255-258 (1993); Bruggermann, et al., *Year in Immuno.* 7:33 (1993). Human antibodies can also be produced in phage-display libraries (Hoogenboom, et al., *J. Mol. Biol.* 227:381 (1991); Marks, et al., *J. Mol. Biol.* 222:581 (1991).

For diagnostic applications, anti-AL-1 antibodies typically will be labeled with a detectable moiety. The detectable moiety can be any one which is capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as  $^3H$ ,  $^{14}C$ ,  $^{32}P$ ,  $^{35}S$ , or  $^{125}I$ , a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin; radioactive isotopic labels, such as, e.g.,  $^{125}I$ ,  $^{32}P$ ,  $^{14}C$ , or  $^3H$ , or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase.

Any method known in the art for separately conjugating the antibody to the detectable moiety may be employed, including those methods described by David, et al., *Biochemistry* 13:1014-1021 (1974); Pain, et al., *J. Immunol. Meth.* 40:219-231 (1981); and Bayer, et al., *Meth. Enz.* 184:138-163 (1990).

The anti-AL-1 antibodies may be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays. Zola, Monoclonal Antibodies: A Manual of Techniques, pp.147-158 (CRC Press, Inc., 1987).

Competitive binding assays rely on the ability of a labeled standard (e.g., AL-1 or an immunologically reactive portion thereof) to compete with the test sample analyte (AL-1) for binding with a limited amount of antibody. The amount of AL-1 in the test sample is inversely proportional to the amount of standard that becomes bound to the antibodies. To facilitate determining the amount of standard that becomes bound, the antibodies generally are insolubilized before or after the competition, so that the standard and analyte that are bound to the antibodies may conveniently be separated from the standard and analyte which remain unbound.

Sandwich assays involve the use of two antibodies, each capable of binding to a different immunogenic portion, or epitope, of the protein to be detected. In a sandwich assay, the test sample analyte is bound by a first antibody which is immobilized on a solid support, and thereafter a second antibody binds to the analyte, thus forming an insoluble three part complex. David, et al., U.S. Pat No. 4,376,110. The second antibody may itself be labeled with a detectable moiety (direct sandwich assays) or may be measured using an anti-immunoglobulin antibody that is labeled with a detectable moiety (indirect sandwich assay). For example, one type of sandwich assay is an ELISA assay, in which case the detectable moiety is an enzyme.

The anti-AL-1 antibodies of the invention also are useful for *in vivo* imaging, wherein an antibody labeled with a detectable moiety is administered to a host, preferably into the bloodstream, and the presence and location of the labeled antibody in the host is assayed. This imaging technique is useful in the staging and treatment of various neurological disorders. The antibody may be labeled with any moiety that is detectable in a host, whether by nuclear magnetic resonance, radiology, or other detection means known in the art.

Neutralizing anti-AL-1 antibodies are useful as antagonists of AL-1. The term "neutralizing anti-AL-1 antibody" as used herein refers to an antibody that is capable of specifically binding to AL-1, and which is capable of substantially inhibiting or eliminating the functional activity of AL-1 *in vivo* or *in vitro*. Typically a neutralizing antibody will inhibit the functional activity of AL-1 at least about 50%, and preferably greater than 80%, as determined, for example, by an *in vitro* receptor binding assay, or *in vitro* axon bundling assay, such as described in Example 8.

Other AL-1 antagonists are prepared using AL-1 receptor proteins, such as REK7. One example of an AL-1 antagonist is the REK7-IgG chimeric protein described herein. Another example of an AL-1 antagonist is a soluble form of an AL-1 receptor, which comprises the extracellular domain or the receptor substantially free of the transmembrane domain. The soluble form of the receptor can be used directly as an antagonist, or the receptor can be used to screen for small molecules that would antagonize AL-1 activity.

AL-1 is believed to be useful in promoting the development, maintenance, or regeneration of neurons *in vivo*, including central (brain and spinal chord), peripheral (sympathetic, parasympathetic, sensory, and enteric neurons), and motoneurons. Accordingly, AL-1 may be utilized in methods for the diagnosis and/or treatment of a variety of neurologic diseases and disorders.

In various embodiments of the invention, purified AL-1 can be administered to patients in whom the nervous system has been damaged by trauma, surgery, stroke, ischemia, infection, metabolic disease, nutritional

deficiency, malignancy, or toxic agents, to promote the survival or growth of neurons. For example, AL-1 can be used to promote the survival or growth of motoneurons that are damaged by trauma or surgery. Also, AL-1 can be used to treat motoneuron disorders, such as amyotrophic lateral sclerosis (Lou Gehrig's disease), Bell's palsy, and various conditions involving spinal muscular atrophy, or paralysis. AL-1 can be used to treat human neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, epilepsy, multiple sclerosis, Huntington's chorea, Down's Syndrome, nerve deafness, and Meniere's disease. AL-1 can be used as cognitive enhancer, to enhance learning particularly in dementias or trauma, since they can promote axonal outgrowth and synaptic plasticity, particularly of hippocampal neurons that express REK7 and cortical neurons that express AL-1. Alzheimer's disease, which has been identified by the National Institutes of Aging as accounting for more than 50% of dementia in the elderly, is also the fourth or fifth leading cause of death in Americans over 65 years of age. Four million Americans, 40% of Americans over age 85 (the fastest growing segment of the U.S. population), have Alzheimer's disease. Twenty-five percent of all patients with Parkinson's disease also suffer from Alzheimer's disease-like dementia. And in about 15% of patients with dementia, Alzheimer's disease and multi-infarct dementia coexist. The third most common cause of dementia, after Alzheimer's disease and vascular dementia, is cognitive impairment due to organic brain disease related directly to alcoholism, which occurs in about 10% of alcoholics. However, the most consistent abnormality for Alzheimer's disease, as well as for vascular dementia and cognitive impairment due to organic brain disease related to alcoholism, is the degeneration of the cholinergic system arising from the basal forebrain (BF) to both the cortex and hippocampus (Bigl *et al.* in Brain Cholinergic Systems, M. Steriade and D. Biesold, eds., Oxford University Press, Oxford, pp.364-386 (1990)). And there are a number of other neurotransmitter systems affected by Alzheimer's disease (Davies *Med. Res. Rev.* 3:221 (1983)). However, cognitive impairment, related for example to degeneration of the cholinergic neurotransmitter system, is not limited to individuals suffering from dementia. It has also been seen in otherwise healthy aged adults and rats. Studies that compare the degree of learning impairment with the degree of reduced cortical cerebral blood flow in aged rats show a good correlation (Berman *et al. Neurobiol. Aging* 9:691 (1988)). In chronic alcoholism the resultant organic brain disease, like Alzheimer's disease and normal aging, is also characterized by diffuse reductions in cortical cerebral blood flow in those brain regions where cholinergic neurons arise (basal forebrain) and to which they project (cerebral cortex) (Lofti *et al., Cerebrovasc. and Brain Metab. Rev.* 1:2 (1989)).

Further, AL-1 can be used to treat neuropathy, and especially peripheral neuropathy. "Peripheral neuropathy" refers to a disorder affecting the peripheral nervous system, most often manifested as one or a combination of motor, sensory, sensorimotor, or autonomic neural dysfunction. The wide variety of morphologies exhibited by peripheral neuropathies can each be attributed uniquely to an equally wide number of causes. For example, peripheral neuropathies can be genetically acquired, can result from a systemic disease, or can be induced by a toxic agent. Examples include but are not limited to distal sensorimotor neuropathy, or autonomic neuropathies such as reduced motility of the gastrointestinal tract or atony of the urinary bladder. Examples of neuropathies associated with systemic disease include post-polio syndrome; examples of hereditary neuropathies include Charcot-Marie-Tooth disease, Refsum's disease, Abetalipoproteinemia, Tangier disease, Krabbe's disease, Metachromatic leukodystrophy, Fabry's disease, and Dejerine-Sottas syndrome; and examples

of neuropathies caused by a toxic agent include those caused by treatment with a chemotherapeutic agent such as vincristine, cisplatin, methotrexate, or 3'-azido-3'-deoxythymidine.

In still further embodiments of the invention, AL-1 antagonists, and especially anti-AL-1 antibodies, can be administered to patients suffering from neurologic diseases and disorders characterized by excessive production or activity of AL-1. AL-1 antagonists can be used in the prevention of aberrant regeneration of sensory neurons such as may occur post-operatively, or in the selective ablation of sensory neurons, for example, in the treatment of chronic pain syndromes.

The development of a vascular supply, angiogenesis, is essential for the growth, maturation, and maintenance of normal tissues, including neuronal tissues. It is also required for wound healing and the rapid growth of solid tumors and is involved in a variety of other pathological conditions. Current concepts of angiogenesis, based in large part on studies on the vascularization of tumors, suggest that cells secrete angiogenic factors which induce endothelial cell migration, proliferation, and capillary formation. Numerous factors have been identified which induce vessel formation *in vitro* or *in vivo* in animal models. These include FGF $\alpha$ , FGF $\beta$ , TGF- $\alpha$ , TNF- $\alpha$ , VPF or VEGF, monobutyrin, angiotropin, angiogenin, hyaluronic acid degradation products, and more recently, B61 for TNF- $\alpha$ -induced angiogenesis (Pandey et al. (1995) *Science* 268:567-569). Inhibitors of angiogenesis include a cartilage-derived inhibitor identified as TIMP, PF-4, thrombospondin, laminin peptides, heparin/cortisone, minocycline, fumagillin, difluoromethyl ornithine, sulfated chitin derivatives, and B61 antibody. The major development of the vascular supply occurs during embryonic development, at ovulation during formation of the corpus luteum, and during wound and fracture healing. Many pathological disease states are characterized by augmented angiogenesis including tumor growth, diabetic retinopathy, neovascular glaucoma, psoriasis, and rheumatoid arthritis. During these processes normally quiescent endothelial cells which line the blood vessels sprout from sites along the vessel, degrade extracellular matrix barriers, proliferate, and migrate to form new vessels. Angiogenic factors, secreted from surrounding tissue, direct the endothelial cells to degrade stromal collagens, undergo directed migration (chemotaxis), proliferate, and reorganize into capillaries.

AL-1 can find further use in promoting or enhancing angiogenesis by receptor activation on endothelial or stromal cells. The induction of vascularization is a critical component of the wound healing process. Neovascularization, also known as angiogenesis, is a complex process involving several sequential steps including basement membrane degradation, endothelial cell mobilization and proliferation, vessel canalization, and new basement membrane formation (Mantovani, *Int. J. Cancer* 25:617 (1980)). Vascularization ensures that proliferating and differentiating fibroblasts are supplied with nutrients and oxygen, and that elements of humoral and cellular immunity are delivered to sites of potential bacterial infection. It is desirable to induce neovascularization as early as possible in the course of wound healing, particularly in the case of patients having conditions that tend to retard wound healing, e.g. burns, decubitus ulcers, diabetes, obesity and malignancies. Even normal post-surgical patients will be benefited if they can be released from hospital care at any earlier date because of accelerated wound healing. This invention provides novel compositions and methods for modulating angiogenesis. A patient bearing a wound can be treated by applying an angiogenically active dose of an AL-1 compound to the wound. This facilitates the neovascularization of surgical incisions, burns, traumatized tissue,

skin grafts, ulcers and other wounds or injuries where accelerated healing is desired. In individuals who have substantially impaired wound healing capacity, thereby lack the ability to provide to the wound site endogenous factors necessary for the process of wound healing, the addition of exogenous AL-1 and compositions of the invention enable wound healing to proceed in a normal manner. The proteins of the present invention are expected to accelerate the healing process in a broad spectrum of wound conditions. Novel topical compositions containing an AL-1 compound are provided for use in the inventive method, as are novel articles such as sutures, grafts and dressings containing an AL-1 compound. The term "wound" is defined herein as any opening in the skin, mucosa or epithelial linings, most such openings generally being associated with exposed, raw or abraded tissue. There are no limitations as to the type of wound or other traumata that can be treated in accordance with this invention, such wounds including (but are not limited to): first, second and third degree burns (especially second and third degree); surgical incisions, including those of cosmetic surgery; wounds, including lacerations, incisions, and penetrations; and ulcers, e.g., chronic non-healing dermal ulcers, including decubital ulcers (bed-sores) and ulcers or wounds associated with diabetic, dental, hemophilic, malignant and obese patients. Furthermore, normal wound-healing may be retarded by a number of factors, including advanced age, diabetes, cancer, and treatment with anti-inflammatory drugs or anticoagulants, and the proteins described herein may be used to offset the delayed wound-healing effects of such treatments.

Although the primary concern is the healing of major wounds by neovascularization, it is contemplated that an AL-1 compound may also be useful for minor wounds, and for cosmetic regeneration of epithelial cells. Preferably, the wounds to be treated are burns and surgical incisions, whether or not associated with viral infections or tumors. In most cases wounds are not the result of a tumor or a viral infection and ordinarily they do not include tumor cells.

AL-1 is preferably delivered to wounds by topical application, "topical" in this context meaning topical to the wound, and does not necessarily refer to epidermal application. When applied topically, the AL-1 compound is usually combined with other ingredients, such as carriers and/or adjuvants. There are no limitations on the nature of such other ingredients, except that they must be pharmaceutically acceptable, efficacious for their intended administration, and cannot degrade or inactivate AL-1. AL-1 is applied to burns in the form of an irrigant or salve, and if so then in an isotonic solution such as physiological saline solution or D5W. AL-1 is particularly useful in accelerating the growth and survival of skin grafts applied to burns. Ordinarily, an AL-1-containing composition is impregnated into the grafts or adherently coated onto the face of the graft, either on the side of the graft to be applied to the burn or on the exterior side of the graft. AL-1 also is included in burn debridement salves which contain proteases so long as the debridement enzyme does not proteolytically inactivate the AL-1.

AL-1 is impregnated into surgical articles in accordance with this invention, such articles being defined as items to be contacted with wounds which articles are typically water adsorbent or hydratable and which have a therapeutic utility in treating wounds. Examples of surgical articles are dressings, sutures, pledgets, skin grafting films (including living skin grafts as well as collagen-containing membranes or synthetic skin substitutes) and the like as will be known to the clinician. Dressings for use herein generally comprise water adsorbent laminates containing AL-1 to be adherently placed into contact with wounds. Improved dressings

for use with AL-1 as described herein preferably will have a membrane such as a dialysis membrane interposed between the wound surface and the adsorbent substance in the dressing, the membrane containing pores sufficiently small for AL-1 to diffuse into the wound but not sufficiently large for epithelial cells to penetrate into the adsorbent. The degree of adsorbency will vary considerably and in fact dressings are included herein which are nonadsorbent, i.e., the AL-1 is deposited or stored in an aqueous reservoir which is used to irrigate the wound on a continuous or intermittent basis.

AL-1 also is formulated into ointments or suspensions, preferably in combination with purified collagen, in order to produce semisolid or suspension vehicles. Conventional oleaginous formulations containing AL-1 are useful as salves. Such AL-1 carriers and formulations release AL-1 on a sustained basis at the wound, thereby serving to create a chemotactic gradient that directionally orients neovascularization, e.g. into a skin graft. Sustained release formulations for AL-1 include semipermeable polymer matrices in the form of shaped articles, e.g. films, or microcapsules. Implantable sustained release matrices include copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman et al., *Biopolymers* 22(1):547-556 (1983)), poly (2-hydroxyethyl-methacrylate) (Langer et al., *J. Biomed. Mater. Res.* 15:167-277 (1981) and Langer *Chem. Tech.* 12:98-105 (1982)), ethylene vinyl acetate (Langer et al., *Id.*), or poly-D-(-)-3-Hydroxybutyric acid (EP 133,988A). These formulations may function as bioerodible matrices or as stable sources for the passive diffusion of AL-1.

Sustained release AL-1 compositions for contact with wounds also include liposomally entrapped AL-1. Liposomes containing AL-1 are prepared by methods known per se: DE 3,218,121A; Epstein et al., *Proc. Natl. Acad. Sci. USA* 82:3688-3692 (1985); Hwang et al. *Proc. Natl. Acad. Sci. USA* 77:4030-4034 (1980); EP 52322A; EP 36676A; EP 88046A; EP 143949A; EP 142641A; Japanese patent application 83-118008; U.S. patents 4,485,045 and 4,544,545; and EP 102,324A. Ordinarily the liposomes are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. % cholesterol, the selected proportion being adjusted for the optimal rate of AL-1 leakage.

AL-1 is formulated with other ingredients such as carriers and/or adjuvants, e.g. albumin, nonionic surfactants and other emulsifiers. There are no limitations on the nature of such other ingredients, except that they must be pharmaceutically acceptable, efficacious for their intended administration, and cannot degrade the activity of the active ingredients of the compositions. Suitable adjuvants include collagen or hyaluronic acid preparations, fibronectin, factor XIII, or other proteins or substances designed to stabilize or otherwise enhance the active therapeutic ingredient(s).

AL-1 optionally is supplied with other known angiogenic agents such as heparin, which has been shown to accelerate the healing of thermal burns, TNF, TGF- $\alpha$ , TGF- $\beta$ , fibroblast growth factor, epidermal growth factor, B61, angiogenin, platelet factor 4, insulin, PDGF, and angiogenesis factor and the angiogenic activity of the combinations observed for synergistic effects. AL-1 optionally also is combined with an IFN, e.g. IFN- $\gamma$ , and other cytokines, or may be free of interferons such as IFN- $\gamma$ . Where such cytokines or known angiogenic agents are species-specific, the appropriate cytokine or agent will be selected for the species to be treated.

Animals or humans are treated in accordance with this invention. It is possible but not preferred to treat an animal of one species with AL-1 of another species. A preferred AL-1 for use herein is soluble AL-1-IgG.

The amount of AL-1 to be contacted with the wound depends upon a great number of variables that will be taken into account by the clinician, including the presence of other angiogenic agents in the AL-1 formulations, the nature of the wound to be treated, the condition of the patient, the AL-1 formulation selected, the neovascularizing activity of the molecular species of AL-1 chosen and the route of administration. Lesser amounts of AL-1 typically are administered when the AL-1 is formulated into a sustained release vehicle, e.g. dressing or ointment, and when the AL-1 is administered by direct topical contact rather than impregnated into a bandage or dressing. Concentrations in the range of 0.10 ng/ml - 100 ug/ml may be used. The typical topical formulation will be capable of delivering a concentration of AL-1-IgG, or equivalent, at the neovascularization target site (for example, a skin graft) in a range of about from 0.10 ng/ml to 10000 ng/ml, more preferably 0.20 ng/ml to 1000 ng/ml, and even more preferably 0.25 ng/ml to 350 ng/ml, although this therapeutic dose range is subject to considerable variation as noted above. Delivery of concentrations outside of this range may offer certain of the benefits of AL-1 neovascularization, but the clinician will be expected to monitor dosages in order to optimize performance of AL-1 in wound healing. It also should be noted that the weight amount will vary for other AL-1 variants and forms if their molecular weight and/or angiogenic potency differ from that of AL-1-IgG. Potency differences are easily determined by comparing the degree of neovascularization achieved with the candidate AL-1 and AL-1-IgG in any of the assays set forth in the Examples herein.

Accordingly, a method for accelerating the neovascularization of a wound is provided that includes the step of applying to the wound an angiogenically effective dose of a composition comprising tumor necrosis factor. The composition can be applied topically by direct contact with the wound, particularly when the wound is a fresh surgical incision. In a preferred embodiment the composition further comprises collagen or a synthetic skin substitute. The caregiver may further administer to the wound a growth factor, an antibiotic, a debridement agent, and/or angiogenin. A preferred composition for the debridement of burns contains an AL-1 compound and a proteolytic enzyme which does not inactivate the neovascularizing activity of AL-1. The therapeutic compositions can be reapplied at one-to-several-day-intervals until healing is complete.

Therapeutic options for patients with vascular disease, particularly vascular obstructive disease, are sometimes limited. Such patients are often refractory to conservative measures and typically unresponsive to drug therapy (Takeshita et al., *J. Clin. Invest.* 93:662-670 (1994)). When vascular obstruction is lengthy and/or widespread, nonsurgical revascularization may not be feasible. Surgical therapy, consisting of arterial bypass and/or amputation, may be complicated by a variable morbidity and mortality, and is often dependent for its efficacy upon short- and long-term patency of the conduit used. Therapeutic angiogenesis constitutes an alternative treatment strategy for such patients. The present invention provides methods for enhancing angiogenesis in a mammal comprising administering to the mammal an effective amount of AL-1. The AL-1 alone may be administered to the mammal, or alternatively, may be administered to the mammal in combination with other therapies and/or pharmacologic agents. In particular AL-1 finds use in patients suffering from vascular insufficiency or limb ischemia secondary to arterial occlusive disease. The effects of AL-1 proteins of the invention on angiogenesis can be tested, for example, in a rabbit model of hindlimb ischemia. This rabbit

model was designed to simulate ischemia characteristics of patients with severe lower extremity arterial occlusive disease. (Takeshita et al., *supra*) and is performed essentially as described in Takeshita et al., *supra*. Measurements of calf blood pressure (BP) index; angiographic score of collateral formation; intravascular Doppler-wire analysis of blood flow; and microsphere-based analysis of muscle perfusion at rest and during stress are performed.

AL-1 antagonists can find use in inhibiting, preventing or treating pathological angiogenesis, such as during tumor vascularization. Tumor neovascularization is a vital stage in the growth of solid tumors (Polverini, P.J. et al. *Lab. Invest.* 51:635-642 (1985)). The progressive growth of solid tumors is strictly dependent on their ability to attract new blood vessels that will supply them with oxygen and essential nutrients (Bouck, *Cancer Cells* 2(6):179-185 (1990)). Angiogenesis has been shown to precede or accompany malignancy. In the absence of neovascularization the size of tumor grafts becomes limited. When angiogenesis is absent, tumors tend to remain dormant. Therefore, angiogenic activity has been directly correlated with tumor growth. AL-1 antagonist compositions and methods of the invention can modulate (e.g., prevent or reduce) new capillary growth into tumors.

A variety of non-neoplastic diseases, previously thought to be unrelated, can be considered "angiogenic diseases" because they are dominated by the pathological growth of capillary blood vessels. These diseases include diabetic retinopathy, arthritis, hemangiomas, psoriasis, and ocular neovascularization. AL-1 antagonist compositions and methods of the invention can be used to treat these conditions.

Vascularization also plays a critical role in chronic inflammatory conditions such as rheumatoid arthritis (Koch, A.E. et al. *Arthr. Rheum.* 29:471-479 (1986)). Rheumatoid arthritis ("RA") is a chronic heterogeneous disorder in which a variety of etiological agents may be responsible for initiating a series of events leading to inflammation in multiple joints. The cause of the disease remains unknown, although by analogy with other forms of arthritis such as that accompanying Lyme disease, it has been postulated that infection with as yet unidentified bacteria or viruses in a genetically susceptible host is an initiating event. Persistence could result from the presence of viral or bacterial antigens that generate an immune response or cross-react with host tissues together with amplification effects of cellular products of the host. While many patients have systemic manifestations in RA, many of the most serious consequences of RA stem from its effects on articular connective tissues, which are characterized by alterations of the synovial membrane with proliferation of lining cells and infiltration by chronic inflammatory cells. Erosions of bone occur in areas contiguous with the inflammatory cell mass as well as in regions adjacent to bone marrow distant from the inflammation. The bone erosions are probably produced through induction of differentiation and activation of osteoclast progenitors. The erosion of soft connective tissues, e.g., cartilage, joint capsules, tendons, and ligaments, results from direct release of proteolytic enzymes from cells of the inflammatory cell mass or from polymorphonuclear leukocytes that are typically abundant in rheumatoid synovial fluids, although rare in the synovial membrane. See, for example, Harris, in W. N. Kelley et al., eds., *Textbook of Rheumatology*. W.B. Saunders, Philadelphia, pp. 886-915 (1985); Dayer and Krane, *Clin. Rheum. Dis.*, 4: 517-537 (1978); Krane, in *Arthritis and Allied Conditions. A textbook of Rheumatology*, ed. by D.J. McCarty, pp. 593-604. Lea and Febiger, Philadelphia (1985); and Krane et al., *Lymphokines* 7:75-136 (1982). Therapy for RA depends on the



stage of the disease. Stage 1, where a postulated antigen is presented to T-cells with no obvious arthritic symptoms, is not treated. Stage 2 involves T-cell and B-cell proliferation and angiogenesis in synovial membrane, resulting in malaise, mild joint stiffness, and swelling. During Stage 3, neutrophils accumulate in synovial fluid and synovial cells proliferate without polarization or invasion of cartilage, resulting in joint pain and swelling, morning stiffness, malaise, and weakness. Current therapy for Stages 2 and 3 includes bed rest, application of heat, supplemental icosapentaenoic and docosahexanoic acid, and drugs. Nonsteroidal anti-inflammatory drugs, including aspirin, continue to be the foundation of drug therapy in treating Stages 2 and 3 of the disease. Those anti-inflammatory drugs other than aspirin include indomethacin, phenylbutazone, phenylacetic acid derivatives such as ibuprofen and fenoprofen, naphthalene acetic acids (naproxen), pyrrolealkanoic acid (tometin), indoleacetic acids (sulindac), halogenated anthranilic acid (meclofenamate sodium), piroxicam, zomepirac, and diflunisal. Second-line drugs for RA Stages 2 and 3 include anti-malarial drugs such as hydroxychloroquine, sulfasalazine, gold salts, and penicillamine, and low-dose methotrexate. These alternatives frequently produce severe side effects, including retinal lesions and kidney and bone marrow toxicity. The irreversible destruction of cartilage occurs in Stage 4 of the disease. Currently available drugs and treatments include total lymphoid irradiation, high-dose intravenous methylprednisolone, and cyclosporine. Cyclosporine is nephrotoxic and the other treatments exert substantial toxicity as well. As a result, such immunosuppressive agents heretofore have been used only in the treatment of severe and unremitting RA. Other possible therapeutic drugs for Stage 4 of RA include cyclic oligosaccharides (cyclodextrins), which, when combined with a noninflammatory steroid (cortexolone), inhibit angiogenesis *in vivo*. Folkman et al., *Science* 243:1490-1493 (1989). Antibodies against crucial components of the early phase of the immune response include anti-Class II MHC antibodies (Gaston et al., *Arthritis Rheum.* 31:21-30 (1988); Sany et al., *Arthritis Rheum.* 25:17-24 (1982)), anti-interleukin-2 receptor antibodies (Kyle et al., *Ann. Rheum. Dis.*, 48:428-429 (1989)), anti-CD4 antibodies (Herzog et al., *J. Autoimmun.*, 2:627-642 (1989); Walker et al., *J. Autoimmun.*, 2:643-649 (1989)), and antithymocyte globulin (Shmerling and Trentham, *Arthritis Rheum.*, 32:1495-1496 (1989)). The last three of these drugs have been used in patients with RA. The present invention provides compositions and methods that down-regulate inflammatory and proliferative pathways in RA by modulating the associated angiogenesis. The compositions contain an angiogenesis-modulating effective amount of an AL-1 antagonist. The compositions can further comprise other angiogenesis-modulating agents, particularly agents for treating RA as discussed above or agents for treating tumors. The methods involve the step of administering an angiogenesis-modulating effective amount of an AL-1 antagonist to a mammal in need of such treatment. By "modulating" in the context of conditions in which angiogenesis is undesirable is meant blocking, inhibiting, preventing, reversing, or reducing angiogenesis, or preventing further progression of angiogenesis.

There seems to be little or no biochemical difference between angiogenic peptides expressed by tumors and those expressed by normal tissues. Nor are there any morphological differences between the new capillaries that respond to a malignancy and the capillary growth that occurs during physiological neovascularization (Folkman et al. *Science* 235:442-447 (1987)). As there is no qualitative difference between the angiogenic capabilities of nonmalignant and malignant diseases, results from normal and malignant vascularization assays can be easily compared, and progress can be made in either area independently of the system of investigation

used (Paweletz et al., *Critical Reviews in Oncology/Hematology* 9(3):197-198 (1989)). Thus, the invention relates to a method comprising inhibiting angiogenesis by administering an effective amount of AL-1 antagonist. The invention comprises a method for the treatment of angiogenesis-dependent diseases by administering an effective amount of AL-1 antagonist to a mammal. Angiogenesis dependent diseases include, but are not limited to diabetic retinopathy, arthritis, tumor growth and metastasis, neovascular glaucoma, retinopathy of prematurity, senile macular degeneration, and hypergeneration of scars after wound healing. The pharmaceutical composition of the present invention exhibit therapeutic properties and preventative or inhibitive properties against diseases associated with angiogenesis, for example, inflammatory diseases (e.g., rheumatoid arthritis), diabetic retinopathy, tumors such as malignant tumors (e.g., cancer such as mastocarcinoma, hepatoma, colic carcinoma, Kaposi's sarcoma, lung carcinomas and other epithelial carcinomas).

Numerous methods, *in vitro* and *in vivo*, are available to screen candidate AL-1 or AL-1 antagonists compounds for angiogenic or angiogenesis-inhibiting properties. Several *in vitro* assays of endothelial cell growth, migration, and capillary tube formation are known and can be used with the compounds of the invention, particularly as initial screening methods for angiogenic or angiostatic substances. Further testing would typically use *in vivo* animal testing. U.S. Patent 5,382,514, which is incorporated herein, describes numerous models for angiogenesis *in vivo*. For example, the corneal pocket assay involves the surgical implantation of polymer pellets containing angiogenic factors in the cornea of larger animals such as rabbits. Since quantitation can be difficult the assay is usually used for preferred candidate compounds. The rabbit ear chamber assay requires the surgical insertion of a glass or plastic viewing device and measurement of capillary migration by microscopy. The rat dorsal air sac assay involves implants of stainless steel chambers containing angiogenic factors. An alginate assay which generates an angiogenic response has been described which involves the injection of tumor cells encased in alginate subcutaneously into mice. The accumulation of hemoglobin in the injected gel is used to quantitate the angiogenic response. A compound can be administered to the chorio-allantoic membranes of aged, typically three-day-aged, fertilized chicken eggs and the appearance of neovascularization after a lapse of time, typically two days is observed (CAM assay; Ausprunk et al. *Am. J. Pathol.* 97:597 (1975)). The neovascularization inhibitory rates are compared with an untreated control group. A more recent assay method involves providing a liquid matrix material which forms a matrix gel when injected into a host; adding an angiogenic agent to the liquid matrix material; injecting the liquid matrix material containing the angiogenic agent into a host to form a matrix gel; recovering the matrix gel from the host; and quantitating angiogenesis of the recovered matrix gel. A variation of this can be used to test for inhibitors of vascularization in a tissue by providing a liquid matrix material which forms a matrix gel when injected into a host; adding an angiogenic inhibiting agent to the liquid matrix material; and injecting the liquid matrix material containing the angiogenic inhibiting agent into a tissue situs of a host to form a matrix gel. This system can be used with compounds of the invention when inducing vascularization in a tissue is desired by providing a liquid matrix material which forms a matrix gel when injected into a host; adding an angiogenic inducing agent to the liquid matrix material; and injecting the liquid matrix material containing the angiogenic inducing agent into a tissue situs of a host to form a matrix gel. In a preferred embodiment, a solution of basement membrane proteins supplemented with fibroblast growth factor and heparin is injected subcutaneously in a host, e.g., a

mouse, where it forms a gel. Sprouts from vessels in the adjacent tissue penetrate into the gel within days connecting it with the external vasculature. Angiogenesis is then quantitated by image analysis of vessels and by measuring the hemoglobin present in the vessels within the gel. This assay method facilitates the testing of both angiogenic and angiostatic agents *in vivo*. In addition, the endothelial cells responding to the angiogenic factors can be recovered *in vitro* for further studies. Preferred compounds have 50-70% inhibition rates, and more preferred compounds show 80-100% inhibition rates. As described herein the angiogenically active proteins of the invention provide use in *in vitro* and *in vivo* screens for compounds that inhibit angiogenesis by measuring inhibition of AL-1 stimulated angiogenesis in the presence and absence of the candidate inhibitor.

Therapeutic formulations of AL-1 and AL-1 antagonists for treating neurologic diseases and disorders and for modulating angiogenesis are prepared by mixing AL-1 or AL-1 antagonist, e.g., anti-AL-1 antibody or soluble REK7, having the desired degree of purity, with optional physiologically acceptable carriers, excipients, or stabilizers which are well known. Acceptable carriers, excipients or stabilizers are nontoxic at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronic or polyethylene glycol (PEG).

It may be desirable to adsorb AL-1 onto a membrane, such as a silastic membrane, which can be implanted in proximity to damaged neural tissue, or to incorporate AL-1 into liposomes. PCT Pat. Pub. No. WO 91/04014 (published April 4, 1991). In another embodiment, the AL-1 used for therapeutic effect is AL-1 covalently joined to another protein, such as an immunoglobulin domain (for example, to produce an AL-1-IgG fusion protein). Immunoglobulin fusions, immunoadhesins, are chimeric antibody-like molecules that combine the functional domain(s) of a binding protein (in this case AL-1 or its receptor) with the an immunoglobulin sequence. The immunoglobulin sequence preferably (but not necessarily) is an immunoglobulin constant domain. Immunoglobulins (Ig) and certain variants thereof are known and many have been prepared in recombinant cell culture. For example, see U.S. Patent 4,745,055; EP 256,654; Faulkner et al., *Nature* 298:286 (1982); EP 120,694; EP 125,023; Morrison, *J. Immun.* 123:793 (1979); Köhler et al., *Proc. Nat'l. Acad. Sci. USA* 77:2197 (1980); Raso et al., *Cancer Res.* 41:2073 (1981); Morrison et al., *Ann. Rev. Immunol.* 2:239 (1984); Morrison, *Science* 229:1202 (1985); Morrison et al., *Proc. Nat'l. Acad. Sci. USA* 81:6851 (1984); EP 255,694; EP 266,663; and WO 88/03559. Reassorted immunoglobulin chains also are known. See for example U.S. patent 4,444,878; WO 88/03565; and EP 68,763 and references cited therein. The immunoglobulin moiety in the chimeras of the present invention may be obtained from IgG-1, IgG-2, IgG-3 or IgG-4 subtypes, IgA, IgE, IgD or IgM, but preferably IgG-1 or IgG-3.

Chimeras constructed from a receptor sequence linked to an appropriate immunoglobulin constant domain sequence (immunoadhesins) are known in the art. Immunoadhesins reported in the literature include fusions of the T cell receptor\* (Gascoigne et al., *Proc. Natl. Acad. Sci. USA* 84:2936-2940 (1987)); CD4\* (Capon

et al., *Nature* 337:525-531 (1989); Traunecker et al., *Nature* 339:68-70 (1989); Zettmeissl et al., *DNA Cell Biol. USA* 9:347-353 (1990); Byrn et al., *Nature* 344:667-670 (1990); L-selectin (homing receptor) (Watson et al. *J. Cell. Biol.* 110:2221-2229 (1990); Watson et al., *Nature* 349:164-167 (1991)); CD44\* (Aruffo et al., *Cell* 61:1303-1313 (1990)); CD28\* and B7\* (Linsley et al., *J. Exp. Med.* 173:721-730 (1991)); CTLA-4\* (Lisley et al., *J. Exp. Med.* 174:61-569 (1991)); CD22\* (Stamenkovic et al., *Cell* 66:1133-1144 (1991)); where the asterisk (\*) indicates that the receptor is member of the immunoglobulin superfamily.

The simplest and most straightforward immunoadhesin design combined the binding region(s) of the 'adhesin' protein (in this case AL-1 or REK-7) with the hinge and Fc regions of an immunoglobulin heavy chain. Ordinarily, when preparing chimeras of the present invention, nucleic acid encoding the extracellular domain or a fragment thereof of AL-1 or REK-7 will be fused C-terminally to nucleic acid encoding the N-terminus of an immunoglobulin constant domain sequence, however N-terminal fusions are also possible. Typically, in such fusions the encoded chimeric polypeptide will retain at least functionally active hinge, CH2 and CH3 domains of the constant region of an immunoglobulin heavy chain. Fusions are also made to the C-terminus of the Fc portion of a constant domain, or immediately N-terminal to the CH1 of the heavy chain or the corresponding region of the light chain. The precise site at which the fusion is made is not critical; particular sites are well known and may be selected in order to optimize the biological activity, secretion or binding characteristics of the AL-1 or REK-7 receptor-immunoglobulin chimeras.

In some embodiments, chimeras are assembled as monomers, or hetero- or homo-multimers, and particularly as dimers or tetramers, essentially as illustrated in WO 91/08298. In a preferred embodiment, the AL-1 or REK-7 extracellular domain sequence is fused to the N-terminus of the C-terminal portion of an antibody (in particular the Fc domain), containing the effector functions of an immunoglobulin, e.g. immunoglobulin G<sub>1</sub> (IgG-1). It is possible to fuse the entire heavy chain constant region to the AL-1 or REK-7 extracellular domain sequence. Preferably a sequence beginning in the hinge region just upstream of the papain cleavage site (which defines IgG Fc chemically; residue 216, taking the first residue of heavy chain constant region to be 114, or analogous sites of other immunoglobulins) is used in the fusion. In one embodiment, an AL-1 or REK-7 amino acid sequence is fused to the hinge region and CH2 and CH3 or CH1, hinge, CH2 and CH3 domains of an IgG-1, IgG-2, or IgG-3 heavy chain. The precise site at which the fusion is made is not critical, and the optimal site can be determined by routine experimentation. The immunoglobulin portion can genetically engineered or chemically modified to inactivate a biological activity of the immunoglobulin portion, such as T-cell binding, while retaining desirable properties such as its scaffolding property for presenting AL-1 or REK7 function to an axon or target cell. Chimeras can be assembled as multimers, particularly as homo-dimers or -tetramers. Generally, these assembled immunoglobulins will have known unit structures. A basic four chain structural unit is the form in which IgG, IgD, and IgE exist. A four unit is repeated in the higher molecular weight immunoglobulins; IgM generally exists as a pentamer of basic four units held together by disulfide bonds. IgA globulin, and occasionally IgG globulin, may also exist in multimeric form in serum. In the case of multimer, each four unit may be the same or different. Alternatively, the AL-1 or REK-7 extracellular domain sequences can be inserted between immunoglobulin heavy chain and light chain sequences such that an immunoglobulin comprising a chimeric heavy chain is obtained. In this

embodiment, the sequences are fused to the 3' end of an immunoglobulin heavy chain in each arm of an immunoglobulin, either between the hinge and the CH2 domain, or between the CH2 and CH3 domains. See Hoogenboom, H. R. et al., *Mol. Immunol.* 28:1027-1037 (1991). The presence of an immunoglobulin light chain is not required in the immunoadhesins of the present invention; an immunoglobulin light chain might be present either covalently associated to a immunoglobulin heavy chain fusion polypeptide, or directly fused to the AL-1 or REK-7 extracellular domain. In the former case, DNA encoding an immunoglobulin light chain is typically coexpressed with the DNA encoding the AL-1- or REK-7-immunoglobulin heavy chain fusion protein. Upon secretion, the hybrid heavy chain and the light chain will be covalently associated to provide an immunoglobulin-like structure comprising two disulfide-linked immunoglobulin heavy chain-light chain pairs.

Preparation of such structures are, for example, disclosed in U.S. Patent No. 4,816,567 issued 28 March 1989. The immunoglobulin sequences used in the construction of the immunoadhesins of the present invention can be from an IgG immunoglobulin heavy chain constant domain. For human immunoadhesins, the use of human IgG1 and IgG3 immunoglobulin sequences is preferred. A major advantage of using IgG1 is that IgG1 immunoadhesins can be purified efficiently on immobilized protein A. In contrast, purification of IgG3 requires protein G, a significantly less versatile medium. However, other structural and functional properties of immunoglobulins should be considered when choosing the Ig fusion partner for a particular immunoadhesin construction. For example, the IgG3 hinge is longer and more flexible, so it can accommodate larger 'adhesin' domains that may not fold or function properly when fused to IgG1. Another consideration may be valency: IgG immunoadhesins are bivalent homodimers, whereas Ig subtypes like IgA and IgM may give rise to dimeric or pentameric structures, respectively, of the basic Ig homodimer unit. For AL-1-Ig or REK-7-Ig immunoadhesins designed for *in vivo* application, the pharmacokinetic properties and the effector functions specified by the Fc region are important as well. Although IgG1, IgG2 and IgG4 all have *in vivo* half-lives of 21 days, their relative potencies at activating the complement system are different. IgG4 does not activate complement, and IgG2 is significantly weaker at complement activation than IgG1. Moreover, unlike IgG1, IgG2 does not bind to Fc receptors on mononuclear cells or neutrophils. While IgG3 is optimal for complement activation, its *in vivo* half-life is approximately one third of the other IgG isotypes. Another important consideration for immunoadhesins designed to be used as human therapeutics is the number of allotypic variants of the particular isotype. In general, IgG isotypes with fewer serologically-defined allotypes are preferred. For example, IgG1 has only four serologically-defined allotypic sites, two of which (G1m and 2) are located in the Fc region; and one of these sites G1m1, is non-immunogenic. In contrast, there are 12 serologically-defined allotypes in IgG3, all of which are in the Fc region; only three of these sites (G3m5, 11 and 21) have one allotype which is nonimmunogenic. Thus, the potential immunogenicity of a  $\gamma$ 3 immunoadhesin is greater than that of a  $\gamma$ 1 immunoadhesin.

In designing the chimeras of the present invention domains that are not required for neurotrophin binding and/or biological activity may be deleted. In such structures, it is important to place the fusion junction at residues that are located between domains, to avoid misfolding. With respect to the parental immunoglobulin, a useful joining point is just upstream of the cysteines of the hinge that form the disulfide bonds between the

two heavy chains. In a frequently used design, the codon for the C-terminal residue of the 'adhesin' part of the molecule is placed directly upstream of the codons for the sequence DKTHTCPPCP of the IgG1 hinge region.

The general methods suitable for the construction and expression of immunoadhesins are the same those disclosed hereinabove with regard to (native or variant) AL-1 or REK7. For example, AL-1-Ig immunoadhesins are most conveniently constructed by fusing the cDNA sequence encoding the AL-1 portion in-frame to an Ig cDNA sequence. However, fusion to genomic Ig fragments can also be used (see, e.g. Gascoigne et al., *Proc. Natl. Acad. Sci. USA* 84:2936-2940 (1987); Aruffo et al., *Cell* 61:1303-1313 (1990); Stamenkovic et al., *Cell* 66:1133-1144 (1991)). The latter type of fusion requires the presence of Ig regulatory sequences for expression. cDNAs encoding IgG heavy-chain constant regions can be isolated based on published sequence from cDNA libraries derived from spleen or peripheral blood lymphocytes, by hybridization or by polymerase chain reaction (PCR) techniques. The cDNAs encoding the 'adhesin' and the Ig parts of the immunoadhesin are inserted in tandem into a plasmid vector that directs efficient expression in the chosen host cells. For expression in mammalian cells pRK5-based vectors (Schall et al., *Cell* 61:361-370 (1990)) and CDM8-based vectors (Seed, *Nature* 329:840 (1989)). The exact junction can be created by removing the extra sequences between the designed junction codons using oligonucleotide-directed deletional mutagenesis (Zoller and Smith, *Nucleic Acids Res.* 10:6487 (1982); Capon et al., *Nature* 337:525-531 (1989)). Synthetic oligonucleotides can be used, in which each half is complementary to the sequence on either side of the desired junction; ideally, these are 36 to 48-mers. Alternatively, PCR techniques can be used to join the two parts of the molecule in-frame with an appropriate vector.

The choice of host cell line for the expression of AL-1-Ig immunoadhesins depends mainly on the expression vector. Another consideration is the amount of protein that is required. Milligram quantities often can be produced by transient transfections. For example, the adenovirus E1A-transformed 293 human embryonic kidney cell line can be transfected transiently with pRK5-based vectors by a modification of the calcium phosphate method to allow efficient immunoadhesin expression. CDM8-based vectors can be used to transfect COS cells by the DEAE-dextran method (Aruffo et al., *Cell* 61:1303-1313 (1990); Zettmeissl et al., *DNA Cell Biol.* (US) 9:347-353 (1990)). If larger amounts of protein are desired, the immunoadhesin can be expressed after stable transfection of a host cell line. For example, a pRK5-based vector can be introduced into Chinese hamster ovary (CHO) cells in the presence of an additional plasmid encoding dihydrofolate reductase (DHFR) and conferring resistance to G418. Clones resistant to G418 can be selected in culture; these clones are grown in the presence of increasing levels of DHFR inhibitor methotrexate; clones are selected, in which the number of gene copies encoding the DHFR and immunoadhesin sequences is co-amplified. If the immunoadhesin contains a hydrophobic leader sequence at its N-terminus, it is likely to be processed and secreted by the transfected cells. The expression of immunoadhesins with more complex structures may require uniquely suited host cells; for example, components such as light chain or J chain may be provided by certain myeloma or hybridoma cell hosts (Gascoigne et al., 1987, *supra*; Martin et al., *J. Virol.* 67, 3561-3568 (1993)).

Immunoadhesins can be conveniently purified by affinity chromatography. The suitability of protein A as an affinity ligand depends on the species and isotype of the immunoglobulin Fc domain that is used in the chimera. Protein A can be used to purify immunoadhesins that are based on human  $\gamma 1$ ,  $\gamma 2$ , or  $\gamma 4$  heavy chains

(Lindmark et al., *J. Immunol. Meth.* 62:1-13 (1983)). Protein G is recommended for all mouse isotypes and for human  $\gamma 3$  (Guss et al., *EMBO J.* 5:1567-1575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. The conditions for binding an immunoadhesin to the protein A or G affinity column are dictated entirely by the characteristics of the Fc domain; that is, its species and isotype. Generally, when the proper ligand is chosen, efficient binding occurs directly from unconditioned culture fluid. One distinguishing feature of immunoadhesins is that, for human  $\gamma 1$  molecules, the binding capacity for protein A is somewhat diminished relative to an antibody of the same Fc type. Bound immunoadhesin can be efficiently eluted either at acidic pH (at or above 3.0), or in a neutral pH buffer containing a mildly chaotropic salt. This affinity chromatography step can result in an immunoadhesin preparation that is >95% pure.

Other methods known in the art can be used in place of, or in addition to, affinity chromatography on protein A or G to purify immunoadhesins. Immunoadhesins behave similarly to antibodies in thiophilic gel chromatography (Hutchens and Porath, *Anal. Biochem.* 159:217-226 (1986)) and immobilized metal chelate chromatography (Al-Mashikhi and Makai, *J. Dairy Sci.* 71:1756-1763 (1988)). In contrast to antibodies, however, their behavior on ion exchange columns is dictated not only by their isoelectric points, but also by a charge dipole that may exist in the molecules due to their chimeric nature.

If desired, the immunoadhesins can be made bispecific, that is, directed against two distinct ligands. Thus, the immunoadhesins of the present invention may have binding specificities for AL-1 and REK-7, or may specifically bind to a AL-1 (or conversely REK7) and to an other determinant specifically expressed on the cells expressing the AL-1 (or the REK7) to which the REK7 (or AL-1) portion of the immunoadhesin structure binds. For bispecific molecules, trimeric molecules, composed of a chimeric antibody heavy chain in one arm and a chimeric antibody heavy chain-light chain pair in the other arm of their antibody-like structure are advantageous, due to ease of purification. In contrast to antibody-producing quadromas traditionally used for the production of bispecific immunoadhesins, which produce a mixture of ten tetramers, cells transfected with nucleic acid encoding the three chains of a trimeric immunoadhesin structure produce a mixture of only three molecules, and purification of the desired product from this mixture is correspondingly easier.

It is apparent that AL-1 antagonists can be prepared or used applying the above guidelines appropriately. For example, a REK7-IgG or anti-AL-1 antibody can be adsorbed onto a membrane, such as a silastic membrane, which can be implanted in proximity to tumors or RA tissue, or can be incorporated into liposomes. PCT Pat. Pub. No. WO 91/04014 (published April 4, 1991).

AL-1 optionally is combined with or administered in concert with other neurotrophic factors to achieve a desired therapeutic effect. For example, AL-1 may be used together with NGF, NT-3, BDNF, NT-4/5, an insulin-like growth factor (e.g., IGF-1, IGF-2, or IGF-3) or another neurotrophic factor to achieve a synergistic stimulatory effect on the growth of sensory neurons, wherein the term "synergistic" means that the effect of the combination of AL-1 with a second substance is greater than that achieved with either substance used individually.

AL-1 and AL-1 antagonists to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration of a solution of AL-1 or anti-AL-1 antibody through sterile filtration membranes. Thereafter, the filtered solution may be placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle. The filtered  
5 solution also may be lyophilized to produce sterile AL-1 or anti-AL-1 antibody in a powder form.

Methods for administering AL-1 and AL-1 antagonists *in vivo* include injection or infusion by intravenous, intraperitoneal, intracerebral, intrathecal, intramuscular, intraocular, intraarterial, or intralesional routes, and by means of sustained-release formulations.

Sustained-release formulations generally consist of AL-1 or AL-1 antagonists and a matrix from which  
10 the AL-1 or AL-1 antagonists are released over some period of time. Suitable matrices include semipermeable polymer matrices in the form of shaped articles, for example, membranes, fibers, or microcapsules. Sustained release matrices may comprise polyesters, hydrogels, polylactides, U.S. Pat. No. 3,773,919, copolymers of L-glutamic acid and gamma ethyl-L-glutamate, Sidman, et al., *Biopolymers*, 22: 547-556 (1983), poly (2-hydroxyethyl-methacrylate), or ethylene vinyl acetate, Langer, et al., *J. Biomed. Mater. Res.* 15: 167-277 (1981);  
15 Langer, *Chem. Tech.* 12:98-105 (1982).

In one embodiment of the invention, the therapeutic formulation comprises AL-1 or AL-1 antagonist entrapped within or complexed with liposomes. For example, AL-1 covalently joined to a glycerophosphatidyl-inositol moiety may be used to form a liposome comprising AL-1. In a further embodiment, the therapeutic formulation comprises cells actively producing AL-1 or AL-1 antagonist. Such cells may be directly introduced  
20 into the tissue of a patient, or may be encapsulated within porous membranes which are then implanted in a patient, in either case providing for the delivery of AL-1 or anti-AL-1 antagonist into areas within the body of the patient in need of increased or decreased concentrations of AL-1. Alternatively, an expression vector comprising AL-1 DNA may be used for *in vivo* transformation of a patient's cells to accomplish the same result.

An effective amount of AL-1 or AL-1 antagonist, e.g., anti-AL-1 antibody, to be employed  
25 therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. A typical daily dosage might range from about 1 µg/kg to up to 100 mg/kg or more, depending on the factors mentioned above. Where possible, it is desirable to determine appropriate dosage ranges first *in vitro*, for example, using assays for neuronal cell  
30 survival or growth which are known in the art, and then in suitable animal models, from which dosage ranges for human patients may be extrapolated. In a specific embodiment of the invention, a pharmaceutical composition effective in promoting the survival or growth of neurons will provide a local GPA concentration *in vivo* of between about 0.1 and 10 ng/ml.

In the treatment of tumors the compositions described herein can be administered subcutaneously or  
35 intramuscularly, for example, and the pharmacological activities of an AL-1 antagonist can be maintained over a long period of time by the sustained-release effect of a composition of the present invention. The number of administrations can therefore be reduced. The composition can also be by directly injecting the composition into a tumor-controlling artery. In the case of treatment of an adult patient having a tumor, the dose of the AL-1



antagonist can be appropriately selected depending upon the kind of tumor, site, size, and kind of AL-1 antagonist. For example, the dose of a protein AL-1 antagonists, particularly an antibody, can be about 0.1 mg to about 500 mg, typically about 1.0 mg to about 300 mg, more typically about 25 mg to about 100 mg. The administration frequency can be appropriately selected depending upon the kind of disease and dosage form.

- 5 In the case of injection into the tumor-controlled artery or tumor itself, frequently repeated injections are not required and a single injection once every one to 4 weeks may be sufficient for the desired therapeutic effects.

In summary, by providing nucleic acid molecules encoding AL-1, the present invention enables for the first time the production of AL-1 by recombinant DNA methods, thus providing a reliable source of sufficient quantities of AL-1 for use in various diagnostic and therapeutic applications. In view of its distinct biological  
10 properties, purified recombinant AL-1 will be especially useful in a variety of circumstances where it is necessary or desirable to assure neuronal function, growth and survival, or angiogenesis, but where other neurotrophic factors or angiogenic agents either cannot be used or are ineffective.

The following examples are offered by way of illustration only and are not intended to limit the invention in any manner. All patent and literature references cited herein are expressly incorporated.

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### EXAMPLE 1

#### Identification and Isolation of REK7 cDNA

To isolate novel growth factors that might act on central nervous system neurons, a search for new tyrosine kinase receptors was made using a polymerase chain reaction (PCR). Degenerate sense and antisense primers were prepared that corresponded to conserved amino acid sequences within the kinase domain of many  
20 receptor tyrosine kinases. The nucleotide sequences of the primers were as follows:

5'- CCCTCGAGGTCGACCA YMIGAYYTIGCIRCIMGIAA - 3' (SEQ. ID NO. 5)

5'- CCCTCGAGGTCGACCA YMIGAYYTIGCITGYMGIAA - 3' (SEQ. ID NO. 6)

5'- CCGCGGTGCGGCCGCCRWAISHCCAIACRTC - 3' (SEQ. ID NO. 7)

These degenerate primers were used to amplify the cDNAs of an adult mouse hippocampal cDNA  
25 library using standard PCR methods, and the resulting amplified cDNAs were subcloned and sequenced. One of these cDNAs, approximately 200 bp in size, encoded a deduced amino acid sequence that appeared to be related to an amino acid sequence within the kinase domains of the eph family of receptor tyrosine kinases. This 200 bp cDNA then was used as a probe to isolate a full-length cDNA from a rat hippocampal cDNA library. Figure 1 shows the nucleotide sequence (SEQ. ID NO. 1) of the full-length cDNA, and the deduced amino acid  
30 sequence (SEQ. ID NO. 2) of the encoded protein, termed REK7.

REK7 appears to be the rat homologue of cek7, and is closely related to ehk-1 and bsk. (Maisonpierre, et al., *Oncogene* 8:3277-3288 (1993); Zhou, et al., *J. Neurosci. Res.* 37:129-143 (1994)). In particular, the REK7 cDNA corresponds to a splice variant of ehk-1 lacking the first of two tandem fibronectin type-III domains.

**EXAMPLE 2****Construction of REK7 Expression Plasmid**

A cDNA encoding REK7 was cloned into the mammalian expression vector pRK7 (PCT Publication No. WO 90/02798, published September 22, 1990), between the XbaI and BamHI restriction endonuclease cleavage sites of pRK7, to produce the expression plasmid pRK-REK7.

**EXAMPLE 3****Production of REK7-IgG Fusion Protein**

DNA encoding a soluble REK7-IgG chimera was constructed by joining DNAs encoding the extracellular domain of REK7 and the F<sub>c</sub> domain of the IgG<sub>1</sub> heavy chain. The DNA sequence encoding the IgG<sub>1</sub> portion of the REK7-IgG chimera was obtained from the -IgG expression plasmid pRKCD4<sub>2</sub>F<sub>c1</sub> (Capon, et al., *Nature* 337:525 (1989); Byrn, et al., *Nature* 344:667 (1990)). That plasmid encodes a hybrid polypeptide consisting of residues 1-180 of the mature human CD4 protein (two N-terminal CD4 variable domains) fused to the portion of the human immunoglobulin IgG<sub>1</sub> protein extending from the aspartic acid residue at position 216 (which is the first residue of the IgG<sub>1</sub> hinge after the cysteine residue involved in heavy-light chain bonding) to amino acid residue 441 (the numbering of residues in IgG<sub>1</sub> is based on amino acid residue 114 being the first residue of the heavy chain constant region (Kabat, et al., *Sequences of Proteins of Immunological Interest*, 4th ed. (1987))).

PCR was used to generate a 600 bp fragment containing sequences encoding the 3'-end of the REK7 extracellular domain (terminating at Gln<sub>462</sub>, the junction between the extracellular domain and the transmembrane domain), joined to sequences encoding the 5'-end of a human IgG<sub>1</sub> Fc domain. The 600 bp fragment was constructed in three steps. First, a DNA fragment containing the 3'-end of the REK7 extracellular domain was amplified using pRK/EK7 as template and 5'-TCTGTGACAGACGATCCTCCC (primer 1, SEQ. ID. NO. 8) and 5'-GCACGGTGGACATGTGTGAGTTTGTCTGGCTTTGATCATTAGATGCTGCAAC (SEQ. ID. NO. 9) as primers. Second, a DNA fragment containing the 5'-end of a human IgG<sub>1</sub> Fc domain was amplified using pRKCD4-IgG as template and 5'-GTTGCAGCATCTAATGATCAAAGCCAGGACAAAACACACATGTCCACCGTGC (SEQ. ID. NO. 10) and 5'-GCACTTGTA CTCTTGCC (primer 2, SEQ. ID. NO. 11) as primers. Finally, the two resulting amplified DNA fragments were mixed together and covalently joined in a third PCR using primers 1 and 2, to produce a 600 bp DNA fragment encoding a REK7-IgG fusion protein.

Since the REK7 cDNA appears to contain sequences that promote rearrangements and deletions during DNA manipulation, assembly of an expression plasmid encoding the full-length REK7-IgG protein was carried out in three steps. First, the 600 bp PCR product (see above) was cut by digestion with KpnI and SacII restriction endonucleases and the resulting KPNI-SacII fragment was gel purified. pRKCD4-IgG<sub>1</sub> was cleaved with HindIII and SacII restriction endonucleases and a 502 bp fragment was isolated. These KpnI-SacII fragment and the 502 bp fragment were ligated into a Bluescript vector (Stratagene, La Jolla, California USA) cleaved with KpnI and HindIII, to produce REK7-IgG Intermediate 1.

Second, REK-IgG Intermediate 1 was cut with KpnI and EcoRI restriction endonucleases and the resulting 1000 bp fragment was isolated. pRK-REK7 was cut with KpnI and Apal restriction endonucleases and a 786 bp fragment was isolated. The 1000 bp fragment and the 786 bp fragment were ligated into a Bluescript vector cut with Apal and EcoRI, to produce REK7-IgG Intermediate 2.

5 Finally, the complete pRKREK-IgG expression plasmid (7004 bp) was assembled by ligation of the three fragments as follows. REK7-IgG Intermediate 2 was cut with PflMI and EcoRI restriction endonucleases and a 1860 bp fragment was isolated. pRK-REK7 was cut with PflMI and PstI restriction endonucleases and a 512 bp fragment was isolated. These two fragments were ligated to the pRK7 vector cut with PstI and EcoRI, to produce pRKREK7-IgG.

10 REK7-IgG was expressed in human embryonic kidney 293 cells (Graham, et al., *J. Gen. Virol.* 36:59 (1977)) and Chinese hamster ovary (CHO) cells by transient transfection using the calcium phosphate precipitation method as described by Capon, et al., *supra*, and Byrn, et al., *supra*. The REK7-IgG chimera was purified to greater than 95% homogeneity from serum free cell culture supernatants by affinity chromatography on immobilized Staphylococcus aureus Protein A as described by Capon, et al., *supra*.

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#### EXAMPLE 4

##### Identification of a Source for REK7 Ligand

In an effort to identify the putative REK7 ligand, REK7-IgG was used to screen cultured cell lines for cell surface expression of REK7-binding activity. Cell lines were assayed by incubation with REK7-IgG and  
20 fluorescent anti-IgG antibody followed by fluorescence-activated cell sorting (FACS).

For example, human breast carcinoma cell line BT20 (American type Culture Collection, Rockville, Maryland, USA) was grown in 50/50 (v/v) F12/DMEM low glucose medium, with 10% fetal calf serum (FCS) and 1 mM glutamine (collectively, growth medium), in a 5% CO<sub>2</sub>. Cells at 80-90% confluence were harvested with 5 mM EDTA in phosphate buffered saline (PBS), counted and resuspended in binding buffer (50/50 (v/v)  
25 F12/DMEM low glucose medium, 5% FCS, and 1mg/ml bovine serum albumin (BSA)) at a cell density of  $5 \times 10^6$  cells/ml. To 1 ml of cells, 1 µg of REK7-IgG was added and incubated for 2 hours at room temperature. The cells then were collected by centrifugation, incubated with a fluorescein-labelled anti-human IgG1-Fc for 1 hour at room temperature, then analyzed by FACS.

The BT20 cell line and the human cervical carcinoma cell line HeLa (American type Culture  
30 Collection, Rockville, Maryland, USA) were found to specifically bind the REK7-IgG. Furthermore, pretreatment of these cells with phosphatidylinositol-specific phospholipase C prior to incubation with REK7-IgG was found to decrease the binding of the REK7-IgG, suggesting the REK7 ligand is linked to the cell membrane by a glycosphosphatidyl-inositol (GPI) anchor.

#### EXAMPLE 5

##### Purification of REK7 Ligand from BT20 Cells

35 BT20 cells were grown to 80% confluence in 150 mm plates, harvested by 5 mM EDTA in PBS, and seeded into 850 mm<sup>2</sup> roller bottles with growth medium. After 1 week of growth the cells were nearly confluent.

The growth medium was removed, the cells washed with PBS, and serum-free growth medium was added. After 5 days, conditioned medium was harvested (12 liters from 72 roller bottles), centrifuged, sterile filtered, concentrated in a 12 kD molecular weight cutoff Amicon filter, and stored at -70°C.

200 ml of BT20 conditioned media, concentrated as described above from 12 liters and frozen, was thawed, centrifuged at 17,000 rpm in a Sorvall SS34 rotor, and filtered through a 0.45 µm filter to clarify the solution. The 200 ml was pumped through a 2.0 ml CD4-IgG - Protein A (CD4-IgG covalently linked to Protein A through IgG moiety) immunoaffinity precolumn and a 1.0 ml REK7-IgG - Protein A (REK7-IgG covalently linked to Protein A through IgG moiety) immunoaffinity column in tandem with a flow rate of 0.5 ml/min. All chromatography was carried out at 4°C. Following one complete passage of the media through both columns, the flow rate was reduced to 0.2 ml/min, and the media was recycled through both columns. Following the loading of the REK7-IgG column, the two columns were separated from each other, washed with 10 column volumes of PBS, and then eluted with four separate washes of 100 mM sodium citrate, pH 3.0 (2 ml each for the CD4-IgG column and 1 ml each for the REK7-IgG column). The first two washes were allowed to immediately flow through the column whereas the last two were incubated for 15 minutes before collection. The eluates were brought to pH 7.4 by the addition of 50 mM potassium phosphate, and an aliquot of each was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Following silver staining of the gel, stained bands of approximately 28,000, 27,000, and 25,000 Daltons were seen from the eluate from the last two washes, that were not seen in eluate obtained by similar treatment of the CD4-IgG precolumn.

#### EXAMPLE 6

##### Protein Sequencing

The 28,000, 27,000, and 25,000 Dalton protein bands that were observed on SDS-PAGE were transferred to a PVDF membrane (Millipore Corporation) by electroblotting, and then subjected to amino acid sequencing using a Applied Biosystems 473A or 470A sequencer. The N-terminus of each of the 28,000 and 25,000 Dalton proteins was blocked, but the N-terminus of the 27,000 Dalton protein gave the following sequence: DRYAVYW(N)SSNPRFQRGDYHIDVXINDY (SEQ. ID NO. 12).

Separate sequence analysis of the three protein bands after cyanogen bromide cleavage or digestion with Lys-C endopeptidase indicated that the 28,000, 27,000, and 25,000 Dalton proteins were related. In particular, the 27,000 Dalton and 25,000 Dalton proteins appeared to be proteolytic processed forms of the 28,000 Dalton protein. Sequence analysis of the 28,000 Dalton band after digestion with Lys-C endopeptidase resulted in two sequences, one of which was nearly identical to the N-terminal sequence of the 27,000 Dalton protein and also had three additional N-terminal residues, AVA. The following internal amino acid sequences were determined for cyanogen bromide and Lys-C fragments of the proteins:

MKTIGVHDRVFDVNDKVENXLEPA (SEQ. ID. NO. 13)

VNFDGYSAXDHTSKGFKRXEXNR (SEQ. ID. NO. 14)

FQLFTPFSLGXEXRXGREXFYISXAIP (SEQ. ID. NO. 15)

KRWEENRP (SEQ. ID. NO. 16)

#### EXAMPLE 7

##### Isolation of REK7 Ligand cDNA

Based on the above protein sequences obtained for the REK7 ligand, two degenerate PCR primers were  
5 synthesized for use in isolating cDNA encoding the REK7 ligand:

5'-CCCTCGAGGTCGACGAYMGITAYGCIGTNTAYTGGA (SEQ. ID. NO. 17)

5'-CCGCGGTGCGGCCGCTCTAGARTAICCRTCRAARTTNACCAT (SEQ. ID. NO. 18).

These degenerate primers were used to amplify the cDNAs of a BT20 cell cDNA library using standard  
PCR methods, and two resulting amplified cDNAs were subcloned and sequenced. These cDNAs,  
10 approximately 180 bp and 135 bp in size, encoded deduced amino acid sequences matching the above amino  
acid sequences determined for the REK7 ligand. The 180 bp fragment was used to screen a human fetal brain  
cDNA library (containing approximately  $2 \times 10^6$  clones) by hybridization under high stringency conditions. The  
nucleotide sequence determined from two independent positive cDNA clones is shown in Figure 2, along with  
the deduced amino acid sequence of the REK7 ligand, termed AL-1.

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#### EXAMPLE 8

##### Biological Activity

Cerebral cortex from postnatal day 2 (P2) Wistar rat pups was dissected in 4°C Hanks balanced salt  
solution (HBSS, calcium and magnesium free) under sterile conditions. After initial trituration with a 10ml  
pipette, cells were dissociated by two passages through an 18 gauge injection cannula attached to a 10ml  
20 syringe. The cell suspension was strained through a 70  $\mu$ m cell strainer (Falcon) and centrifuged at 800 g for  
5 minutes. Pelleted cells were resuspended in 50/50 (v/v) DMEM/F12 with 10% fetal bovine serum, 15mM  
HEPES pH7.4. The cell suspension was plated into 75 cm<sup>2</sup> tissue culture flasks, and placed into a 37° C, 5%  
CO<sub>2</sub> incubator. Cultures were grown to confluence (10-15 days) and the medium was changed every 3 days.  
Once cultures were confluent the flasks were shaken in a rotary shaker at 300 rpm at 37° C for 24 hrs to obtain  
25 purified astrocytes. Media with the suspended cells and debris was replaced and cultures were incubated for  
another 24 hours. Purified astrocyte cultures were trypsinized (0.05% trypsin, 0.025mM EDTA in Hanks) from  
the flasks and then replated into 60mm tissue culture treated dishes (2 dishes per 75cm<sup>2</sup> flask).

Cerebral cortex of embryonic day 16 (E16) rats was dissected in 4° C HBSS under sterile conditions.  
Media was changed to 1 ml of Defined Neuronal Medium ("DNM"; Peterson et al., *Dev. Brain Res.* 48:187-195  
30 (1989) and cortices were triturated by pipeting 15 times through a plastic disposable pipet blue. 9 ml of DNM  
then was added and the cell suspension was strained through a 45 $\mu$ m cell strainer and centrifuged at 800 g for  
5 minutes. Pelleted cells were resuspended in 5 ml of DNM and  $4 \times 10^5$  cells were plated onto 60mm dishes  
containing astrocytes which had been pretreated for 6 hours with either REK7-IgG (30 $\mu$ g/ml), CD4-IgG

(30 µg/ml), or no added IgG (control). Cultures were grown for 4 days and then fixed for 15 minutes with 4% paraformaldehyde in 100 mM Sodium Phosphate buffer, pH 7.4, followed by 3 washes with PBS.

The addition of REK7-IgG to the co-cultures completely prevented the formation of axon bundles. In contrast, the addition of CD4-IgG, and other receptor-IgGs (e.g., TrkA-IgG (Shelton et al. *J. Neurosci.* 15:477-491 (1995), Eph-related HTK-IgG (Bennett et al. *J. Biol. Chem.* 269:14211-14218 (1994)) had no effect on axon bundling. These results indicate a role for REK7 and its ligand, AL-1, in axon fasciculation, which is a crucial step in the development of the nervous system during regeneration following injury.

Consistent with that role, upon incubation of the cultured neuronal cells with labelled anti-REK7 antibodies, there was specific binding of the antibodies to the surface of axonal fibers within axon bundles. A related EPH-family receptor, SEK1 (Gilardi-Hebenstreit et al. *Oncogene* 7:2499-2506 (1992)) failed to bind to the surface of the axonal fibres within the bundles. Furthermore, AL-1 is expressed predominantly on astrocytes in coculture, while REK-7 is expressed on both axon fibers and cell bodies of the cortical neurons.

### EXAMPLE 9

#### Biological Activity: Activation of REK7 by AL-1

When cells expressing REK7 were incubated with AL-1, specific phosphorylation of REK7 was observed, indicating that AL-1 not only binds to REK7 but also activates REK7. Cultured primary cortical neurons were analyzed by immunoprecipitation and immunoblotting with anti-REK7 and anti-phosphotyrosine antibodies. Membrane-bound AL-1 transiently expressed on the surface of transfected 293 cells, strongly activated endogenous REK7 expressed in cortical neurons, as indicated by autophosphorylation of the 135K REK7 polypeptide. In contrast, purified soluble AL-1 only weakly stimulated tyrosine autophosphorylation of REK7 ~2-fold (Winslow et al., *Neuron*, 14:973-981 (1995), which is incorporated by reference). These data (i) confirm the presence of REK7 in cortical neurons, (ii) indicate that AL-1 is an activating ligand, and (iii) demonstrate that membrane-attachment is required for maximal receptor activation as has been seen with other members of this ligand family (Davis et al., *Science* 266:816-819 (1994)).

HEK 293 cells were transfected with an AL-1 cDNA expression plasmid using the calcium phosphate coprecipitation method (Simonsen and Levinson, *Proc. Natl. Acad. Sci.* 80:2495-2499 (1983)). Primary cortical neurons from E16 rats were plated at a density of  $5 \times 10^6$  cells/15 cm dish and cultured for 4 days. These cells were then treated with purified soluble AL-1 (0.1-1 µg/ml) or 293 cells expressing membrane-bound AL-1 for 10 min at 37 °C. Immunoprecipitation of lysates with rabbit anti-REK7 and immunoblotting with mouse anti-phosphotyrosine was essentially as described (Kaplan et al, *Science* 252:554-559 (1991); Kaplan et al., *Nature* 350:158-160 (1991)). Immunoblotted bands were visualised using a horseradish peroxidase-conjugated sheep anti-mouse antibody and the ECL fluorescence detection system (Amersham) as described by the manufacturer.

### EXAMPLE 10

#### Activity of AL-1-IgG Fusion

An AL-1-IgG fusion protein was demonstrated to be a soluble, active form of AL-1. DNA encoding a soluble AL-1-IgG chimera was constructed by joining the DNAs encoding the extracellular domain of AL-1

and the Fc domain of the IgG<sub>1</sub>, similar to the construction of REK7-IgG. AL-1-IgG fusion protein was generated by transfection of 293 cells with the plasmid pRKAL-1-IgG. Conditioned media was collected after 3 days and AL-1-IgG was purified by Protein A chromatography. The ability of AL-1-IgG to activate the REK7 receptor was determined by tyrosine autophosphorylation of rat embryonic (E16) cortical neurons REK7 receptor as described above. Using the same density and number of cortical cells ( $5 \times 10^6$  cells/15 cm plate), 1 ug/ml of AL-1-IgG was compared with 1 ug/ml soluble AL-1, or an equivalent number of 293 cells expressing membrane bound AL-1. No addition, and mock transfected 293 cells were included as controls. AL-1-IgG greatly activated the autophosphorylation of REK7, to a similar extent as membrane-bound AL-1. These results indicate that the soluble AL-1-IgG fusion protein can be used as an agonist for the REK7 receptor in *in vitro* and *in vivo* studies. The inability of soluble AL-1 to activate REK7 autophosphorylation, although the affinity chromatography purification demonstrates binding to the receptor, indicates that the soluble form of AL-1 (and EPH receptor ligands in general toward their cognate receptor) can act as antagonists of REK7.

### EXAMPLE 11

#### Soluble AL-1 is an antagonist of axon bundling

Soluble AL-1 bound to but did not efficiently activate REK7, and soluble AL-1 acted as an antagonist of REK7 receptor activity and blocked axon fasciculation in the cell culture system. The addition of soluble AL-1 to the culture medium of cortical neuron-astrocyte co-cultures specifically prevented the formation of axon bundles, as did REK7-IgG. This result, taken together with the observations that (i) AL-1 is expressed in the co-cultures (predominantly on astrocytes), (ii) REK7 is expressed on both the axons fibres and cell bodies of the cortical neurons and (iii) AL-1 can activate REK7, further implicates AL-1 and REK7 as the molecules involved in axon bundling whose action is inhibited by REK7-IgG.

The effect on axon bundle formation of the anti-REK7 and anti-SEK antibodies, added to the culture medium at 200  $\mu$ g/ml was tested. Neither reagent had any apparent effect on axon bundling in these cultures. Since the anti-REK7 antibodies bind to the axon fibres but fail to block bundle formation, the mere binding to axons is not sufficient to interfere with the bundling process, which further supports that the inhibitory effects of REK7-IgG and soluble AL-1 are due to a specific inhibition of REK7 function.

The following mechanism is not meant to be limiting to the invention. The role of REK7 in axon fasciculation is probably indirect, i.e. REK7 and AL-1 do not themselves function as adhesion molecules but rather are involved in regulating the fasciculation process. Activation of REK7 expressed on the neurons, by AL-1 expressed on the astrocytes, might activate a signaling pathway that promotes fasciculation, for example by up-regulating or activating adhesion molecules. Activation of REK7 may cause growth cone repulsion and collapse, forcing axons together for fasciculation. AL-1 expressed on astrocytes would serve as a repulsive cue to axons driving axons together. This model is compatible with the current view that astrocytes play an important role during development of the CNS, where they are thought to provide a substratum and trophic support for growing axons (Hatten et al., *Semin. Neurosci.* 2:455-465 (1990)).

As taught herein a tyrosine kinase is required for axon fasciculation. Neurons in the developing or regenerating nervous system presumably require two types of factors, those that promote growth and survival,

and those that provide spatial or directional guidance in the establishment of neuronal pathways (Tessier-Lavigne, *Curr. Opin. Genet. Devel.* 4:596-601 (1994)). Tyrosine kinases are known to play a well-established role in the former but have not hitherto been shown to participate in the latter. The teachings herein, combined with the observation that both REK7 and AL-1 are expressed in the brain, indicate a role in the formation of neuronal pathways, a crucial feature of both development and regeneration in the nervous system.

5



## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Genentech, Inc.  
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- (ii) TITLE OF INVENTION: AL-1 Neurotrophic Factor
- (iii) NUMBER OF SEQUENCES: 18
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- (v) COMPUTER READABLE FORM:  
(A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: WinPatin (Genentech)
- (vi) CURRENT APPLICATION DATA:  
(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:  
(A) APPLICATION NUMBER: 08/330128  
(B) FILING DATE: 27-OCT-1994
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(A) APPLICATION NUMBER: 08/486449  
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- (2) INFORMATION FOR SEQ ID NO:1:
- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 4165 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AGCCCCCAGT CCTCTAGACA CTCTGCTTGC TTTTCCCCG CTCCCCGCCG 50  
 CCTTTTTTTT TTTACGGAAA ACATCATCTA GATTTAAGAT GACTCGTCTT 100  
 CTTTAATCTC GTCCGTATCA GCACTGAAGA CTGAAAGGGA ACCTTCACCA 150  
 5 CCACTCCAAC CCTGGTGGGG GCTTAAAAAA AAAAAATAAC AGTTCTAAAA 200  
 AAGAAAGGGG GACCAAAAAA CAGAAAAAGG AAAGTCTTAA GAGGCAAAGG 250  
 AGCGGGACTC GGGACCCTCT GCAGACCCTT GACTCAGCCC ACCCAGGACC 300  
 GTACTAGCCA TCCGAACCTC TAATTCATCT TATCCAACCTG AAAGGGAGGG 350  
 CGGCACAAGC CAGAAGCAAA CTTGCGCGGT CTCTGCGGAT CTGTGATTCC 400  
 10 CACATTGAGA GGGGTCGAGA GCCAGAAGGC ACAGGACCCC ACCAGGAGGA 450  
 GGAGTTCCGC GCCTCTCTCG CCCCTTCCAC CAAGCCTGAA CTTTAGACTG 500  
 AACCACGCGG GACCTAAGAG GCAGAAGAGG GTAGTAGAAA ATGCGGGGCT 550  
 CCGGGCCCCG CGGTGCGGGA CGCCGACGGA CCCAGGGCAG AGGTGGCGGC 600  
 GGCGACACCC CCCGCGTCCC TGCCTCTCTG GCAGGCTGCT ATTCCGCACC 650  
 15 TCTAAAGGGG CCCCTCTGGA CGTGCCTTCT CCTGTGTGCG GCGCTCCGGA 700  
 CCCTTTTGGC CAGCCCCAGT AACGAAGTGA ATTTATTGGA TTCGCGCACT 750  
 GTCCTGGGAG ACCTTGATG GATTGCTTTT CCAAAGAATG GGTGGGAAGA 800  
 GATTGGTGAA GTTGATGAAA ACTATCCCCC CATCCACACC TATCAAGTGT 850  
 GCAAAGTTAT GGAACAGAAT CAGAATAATT GGCTGTTGAC CAGTTGGATC 900  
 20 TCTAACGAAG GTGCTTCCAG AATTTTTATT GAACTCAAGT TTA CTCTGAG 950  
 GGATTGCAAC AGCCTTCCTG GAGGACTGGG GACTTGCAAG GAGACCTTTA 1000  
 ACATGTATTA TTTTGAGTCG GATGATGAGA ATGGGAGAAA TATCAAAGAG 1050  
 AACCAGTACA TCAAGATCGA TACCATTGCT GCTGATGAGA GCTTCACCGA 1100  
 ACTTGACCTT GGAGACCGGG TCATGAAGCT GAATACGGAG GTCAGAGATG 1150  
 25 TAGGACCTCT GAGCAAAAAG GGATTTTATC TTGCTTTCCA AGATGTCGGT 1200  
 GCTTGCAATC CTCTGGTTTC TGTCCGTGTC TACTATAAAA AATGTCCTTC 1250  
 TG TAGTTAGA CATTTGGCTG TTTCCCTGA CACGATCACT GGAGCAGATT 1300  
 CTTCCAGTT GCTAGAGGTG TCAGGCTCCT GCGTCAACCA TTCTGTGACA 1350

GACGATCCTC CAAAAATGCA TTGCAGTGCT GAAGGGGAGT GGCTGGTTCC 1400  
 CATCGGGAAA TGCATGTGCA AGGCCGGATA TGAAGAGAAA AATGGTACCT 1450  
 GTCAAGTGTG CAGACCTGGG TTCTTCAAAG CCTCTCCTCA CAGCCAGACC 1500  
 TGCAGCAAAT GTCCACCTCA CAGTTACACC CATGAGGAAG CTTCCACCTC 1550  
 5 TTGTGTCTGT GAAAAGGATT ATTTCAGGAG GGAATCTGAT CCGCCCACAA 1600  
 TGGCATGCAC AACTCCTTCT CCAGTCACCA ACGTGAAGAA GGGGAAGATC 1650  
 GCAAAGAACA GCATTTCTTT GTCTTGGCAA GAGCCAGATC GCCCCAATGG 1700  
 GATCATCCTG GAGTACGAAA TCAAGTACTT TGAAAAGGAC CAAGAGACCA 1750  
 GCTACACAAT TATCAAGTCT AAAGAGACCA CTATTACGGC AGAGGGCCTG 1800  
 10 AAACCTGCGT CTGTGTATGT CTTCCAAATT CGAGCACGTA CAGCAGCAGG 1850  
 CTACGGCGTC TTCAGTCGAC GGTTCGAGTT TGAAACCACA CCAGTGTGAG 1900  
 TTGCAGCATC TAATGATCAA AGCCAGATTC CCATCATTGC GGTGTCAGTG 1950  
 ACGGTGGGAG TCATCTTGTT GGCAGTGATG ATCGGCTTCC TCCTCAGTGG 2000  
 CAGTTGCTGC GAATGTGGCT GTGGGAGGGC TTCTTCCCTG TGCGCTGTTG 2050  
 15 CCCATCCAAG CCTAATATGG CGGTGTGGCT ACAGCAAAGC AAAGCAGGAT 2100  
 CCAGAAGAGG AAAAGATGCA CTTTCATAAC GGGCACATTA AACTGCCAGG 2150  
 AGTAAGAACG TACATTGATC CACACACCTA CGAAGATCCC ACTCAAGCCG 2200  
 TTCATGAATT TGCCAAGGAG ATCGAGGCCT CATGCATCAC CATTGAGAGA 2250  
 GTTATTGGAG CAGGTGAATT TGGTGAAGTT TGTAGTGGAC GTTTGAAACT 2300  
 20 ACCCGGAAAA AGAGAATTGC CTGTGGCTAT CAAAACCTTT AAAGTAGGCT 2350  
 AACTGAAAA GCAGCGCAGA GATTTTCTGA GTGAAGCGAG TATCATGGGG 2400  
 CAGTTTGATC ATCCAAACAT CATCCATCTA GAAGGCGTTG TGAATAAAG 2450  
 TAAACCTGTG ATGATAGTGA CAGAGTACAT GGAGAATGGC TCCTTAGACA 2500  
 CATTTTTAAA GAAAAACGAT GGCCAGTTCA CTGTGATTCA GCTTGTTGGC 2550  
 25 ATGCTGAGAG GCATCGCTGC AGGAATGAAG TACCTTTCTG ACATGGGCTA 2600  
 CGTGACACAGA GACCTTGCTG CTAGAAACAT CTTAATCAAC AGTAACCTTG 2650  
 TGTGCAAAGT GTCTGACTTT GGACTTTCCA GGGTGCTGGA AGATGATCCT 2700  
 GAGGCAGCCT ATACCACAAG GGGAGGCAAA ATTCCAATCA GATGGACTGC 2750  
 TCCAGAAGCA ATAGCTTTTC GAAAGTTTAC CTCTGCCAGT GATGTCTGGA 2800

GCTATGGAAT CGTAATGTGG GAAGTTGTGT CCTATGGAGA GAGACCGTAC 2850  
TGGGAGATGA CCAATCAGGA TGTGATCAAG GCAGTGGAAG AAGGCTACCG 2900  
CCTGCCAAGC CCCATGGATT GTCCTGCTGC CCTCTATCAA TTAATGCTGG 2950  
ATTGCTGGCA GAAAGATCGC AACAGCAGGC CCAAGTTTGA TGACATAGTC 3000  
5 AACATGCTGG ATAAGCTGAT ACGAAACCCA AGTAGTTTGA AGACACTGGT 3050  
GAACCGCTCA AGCAGGGTAT CTACTTTGTT GGCAGAACAT GGATCTTTGG 3100  
GGTCTGGGGC CTACAGATCA GTAGGTGAAT GGCTAGAAGC AATCAAAATG 3150  
GGCCGGTACA CAGAGATTTT CATGGAAAAT GGATACAGTT CAATGGACGC 3200  
TGTGGCTCAG GTGACCTTGG AGGATTTGAG ACGCCTGGGA GTGACTCTGG 3250  
10 TCGGTCACCA GAAGAAGATC ATGAACAGCC TTCAAGAGAT GAAGGTGCAG 3300  
CTGGTAAACG GGATGGTGCC AGTGTGACCC GCACACGGGT CACTTCTCCA 3350  
AGTGAACAAC TCTGCACTTT GTAAACAGCC CTAAGATTTA TTTTAACAGA 3400  
GAAAGGAAAA TGGGTGGTTC CTAAACCTTT GAAGGCACTT GTCTCATCCT 3450  
TTGACTTATA ATCAACATTT TACTAAAATC TCCAGATCTT CTTCTTAATG 3500  
15 TCTTCGTTTT TTTCACTATG TAAATGTAAC CTGCGAAGAG AGCTAACATG 3550  
AGAAACAACA TCCTATAAAA ACATAGTAAC TAAATCTTTC TCCACTTGCA 3600  
GCCCCTTTCA AAACCTACCAG GGATCGACTT GAAAGGAAAA GTTTTAAAGC 3650  
CATGTGTGGG CAAAGAAACG CTGCATTTTA CTGACGTTTA CTTGAGTTT 3700  
TATTTGTCTG CATAAGTGTA TTGGAGAGCA ATATGATTAG ATTATTTCTT 3750  
20 AAATACAGTT TGTAATTTAA AATGGAATTA CATGTTATAA GTTATAGAAA 3800  
ATAGTTTACA GACATGTTGC CCGGTCAAGG AAAAGTTCAG CACAGGGTGT 3850  
ATATTTATTT TTCTGTGTTA TATAATTTAC TTTTAGTTGC ACTTCTAGAG 3900  
AGTATTAGGC AATGAATGTG TATAGACTGT ATAGTTTGCA ATATACCGAG 3950  
GAATGGACTT AAATTGAAA TGTATGTATA TGTGTGTGTA TGTGCGTATG 4000  
25 TGTGTGTGTG TTTGAATATG TAGATGGTAT TGTTCTGCTT GCCTTTTGTA 4050  
TAGGGTTTTA ATTTTGGCCT CATAAGCAA AGGGTGTTCT AGACTATTTT 4100  
ATGGGTAAGA GGAATAGGAA GCCTTAGACC AAATTTCCCT CAAGTAGGTG 4150  
TCCTTTCTCT CATTT 4165

(2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 928 amino acids

(B) TYPE: Amino Acid

(D) TOPOLOGY: Linear

## 5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

	Met	Arg	Gly	Ser	Gly	Pro	Arg	Gly	Ala	Gly	Arg	Arg	Arg	Thr	Gln	
	1				5					10					15	
	Gly	Arg	Gly	Gly	Gly	Gly	Asp	Thr	Pro	Arg	Val	Pro	Ala	Ser	Leu	
					20					25					30	
10	Ala	Gly	Cys	Tyr	Ser	Ala	Pro	Leu	Lys	Gly	Pro	Leu	Trp	Thr	Cys	
					35					40					45	
	Leu	Leu	Leu	Cys	Ala	Ala	Leu	Arg	Thr	Leu	Leu	Ala	Ser	Pro	Ser	
					50					55					60	
15	Asn	Glu	Val	Asn	Leu	Leu	Asp	Ser	Arg	Thr	Val	Leu	Gly	Asp	Leu	
					65					70					75	
	Gly	Trp	Ile	Ala	Phe	Pro	Lys	Asn	Gly	Trp	Glu	Glu	Ile	Gly	Glu	
					80					85					90	
	Val	Asp	Glu	Asn	Tyr	Pro	Pro	Ile	His	Thr	Tyr	Gln	Val	Cys	Lys	
					95					100					105	
20	Val	Met	Glu	Gln	Asn	Gln	Asn	Asn	Trp	Leu	Leu	Thr	Ser	Trp	Ile	
					110					115					120	
	Ser	Asn	Glu	Gly	Ala	Ser	Arg	Ile	Phe	Ile	Glu	Leu	Lys	Phe	Thr	
					125					130					135	
25	Leu	Arg	Asp	Cys	Asn	Ser	Leu	Pro	Gly	Gly	Leu	Gly	Thr	Cys	Lys	
					140					145					150	
	Glu	Thr	Phe	Asn	Met	Tyr	Tyr	Phe	Glu	Ser	Asp	Asp	Glu	Asn	Gly	
					155					160					165	
	Arg	Asn	Ile	Lys	Glu	Asn	Gln	Tyr	Ile	Lys	Ile	Asp	Thr	Ile	Ala	
					170					175					180	
30	Ala	Asp	Glu	Ser	Phe	Thr	Glu	Leu	Asp	Leu	Gly	Asp	Arg	Val	Met	
					185					190					195	
	Lys	Leu	Asn	Thr	Glu	Val	Arg	Asp	Val	Gly	Pro	Leu	Ser	Lys	Lys	
					200					205					210	
35	Gly	Phe	Tyr	Leu	Ala	Phe	Gln	Asp	Val	Gly	Ala	Cys	Ile	Ala	Leu	
					215					220					225	
	Val	Ser	Val	Arg	Val	Tyr	Tyr	Lys	Lys	Cys	Pro	Ser	Val	Val	Arg	
					230					235					240	
	His	Leu	Ala	Val	Phe	Pro	Asp	Thr	Ile	Thr	Gly	Ala	Asp	Ser	Ser	
					245					250					255	

	Gln	Leu	Leu	Glu	Val	Ser	Gly	Ser	Cys	Val	Asn	His	Ser	Val	Thr	
					260					265					270	
	Asp	Asp	Pro	Pro	Lys	Met	His	Cys	Ser	Ala	Glu	Gly	Glu	Trp	Leu	
					275					280					285	
5	Val	Pro	Ile	Gly	Lys	Cys	Met	Cys	Lys	Ala	Gly	Tyr	Glu	Glu	Lys	
					290					295					300	
	Asn	Gly	Thr	Cys	Gln	Val	Cys	Arg	Pro	Gly	Phe	Phe	Lys	Ala	Ser	
					305					310					315	
10	Pro	His	Ser	Gln	Thr	Cys	Ser	Lys	Cys	Pro	Pro	His	Ser	Tyr	Thr	
					320					325					330	
	His	Glu	Glu	Ala	Ser	Thr	Ser	Cys	Val	Cys	Glu	Lys	Asp	Tyr	Phe	
					335					340					345	
	Arg	Arg	Glu	Ser	Asp	Pro	Pro	Thr	Met	Ala	Cys	Thr	Thr	Pro	Ser	
					350					355					360	
15	Pro	Val	Thr	Asn	Val	Lys	Lys	Gly	Lys	Ile	Ala	Lys	Asn	Ser	Ile	
					365					370					375	
	Ser	Leu	Ser	Trp	Gln	Glu	Pro	Asp	Arg	Pro	Asn	Gly	Ile	Ile	Leu	
					380					385					390	
20	Glu	Tyr	Glu	Ile	Lys	Tyr	Phe	Glu	Lys	Asp	Gln	Glu	Thr	Ser	Tyr	
					395					400					405	
	Thr	Ile	Ile	Lys	Ser	Lys	Glu	Thr	Thr	Ile	Thr	Ala	Glu	Gly	Leu	
					410					415					420	
	Lys	Pro	Ala	Ser	Val	Tyr	Val	Phe	Gln	Ile	Arg	Ala	Arg	Thr	Ala	
					425					430					435	
25	Ala	Gly	Tyr	Gly	Val	Phe	Ser	Arg	Arg	Phe	Glu	Phe	Glu	Thr	Thr	
					440					445					450	
	Pro	Val	Ser	Val	Ala	Ala	Ser	Asn	Asp	Gln	Ser	Gln	Ile	Pro	Ile	
					455					460					465	
30	Ile	Ala	Val	Ser	Val	Thr	Val	Gly	Val	Ile	Leu	Leu	Ala	Val	Met	
					470					475					480	
	Ile	Gly	Phe	Leu	Leu	Ser	Gly	Ser	Cys	Cys	Glu	Cys	Gly	Cys	Gly	
					485					490					495	
	Arg	Ala	Ser	Ser	Leu	Cys	Ala	Val	Ala	His	Pro	Ser	Leu	Ile	Trp	
					500					505					510	
35	Arg	Cys	Gly	Tyr	Ser	Lys	Ala	Lys	Gln	Asp	Pro	Glu	Glu	Glu	Lys	
					515					520					525	
	Met	His	Phe	His	Asn	Gly	His	Ile	Lys	Leu	Pro	Gly	Val	Arg	Thr	
					530					535					540	

	Tyr	Ile	Asp	Pro	His	Thr	Tyr	Glu	Asp	Pro	Thr	Gln	Ala	Val	His
					545					550					555
	Glu	Phe	Ala	Lys	Glu	Ile	Glu	Ala	Ser	Cys	Ile	Thr	Ile	Glu	Arg
					560					565					570
5	Val	Ile	Gly	Ala	Gly	Glu	Phe	Gly	Glu	Val	Cys	Ser	Gly	Arg	Leu
					575					580					585
	Lys	Leu	Pro	Gly	Lys	Arg	Glu	Leu	Pro	Val	Ala	Ile	Lys	Thr	Leu
					590					595					600
10	Lys	Val	Gly	Tyr	Thr	Glu	Lys	Gln	Arg	Arg	Asp	Phe	Leu	Ser	Glu
					605					610					615
	Ala	Ser	Ile	Met	Gly	Gln	Phe	Asp	His	Pro	Asn	Ile	Ile	His	Leu
					620					625					630
	Glu	Gly	Val	Val	Thr	Lys	Ser	Lys	Pro	Val	Met	Ile	Val	Thr	Glu
					635					640					645
15	Tyr	Met	Glu	Asn	Gly	Ser	Leu	Asp	Thr	Phe	Leu	Lys	Lys	Asn	Asp
					650					655					660
	Gly	Gln	Phe	Thr	Val	Ile	Gln	Leu	Val	Gly	Met	Leu	Arg	Gly	Ile
					665					670					675
20	Ala	Ala	Gly	Met	Lys	Tyr	Leu	Ser	Asp	Met	Gly	Tyr	Val	His	Arg
					680					685					690
	Asp	Leu	Ala	Ala	Arg	Asn	Ile	Leu	Ile	Asn	Ser	Asn	Leu	Val	Cys
					695					700					705
	Lys	Val	Ser	Asp	Phe	Gly	Leu	Ser	Arg	Val	Leu	Glu	Asp	Asp	Pro
					710					715					720
25	Glu	Ala	Ala	Tyr	Thr	Thr	Arg	Gly	Gly	Lys	Ile	Pro	Ile	Arg	Trp
					725					730					735
	Thr	Ala	Pro	Glu	Ala	Ile	Ala	Phe	Arg	Lys	Phe	Thr	Ser	Ala	Ser
					740					745					750
30	Asp	Val	Trp	Ser	Tyr	Gly	Ile	Val	Met	Trp	Glu	Val	Val	Ser	Tyr
					755					760					765
	Gly	Glu	Arg	Pro	Tyr	Trp	Glu	Met	Thr	Asn	Gln	Asp	Val	Ile	Lys
					770					775					780
	Ala	Val	Glu	Glu	Gly	Tyr	Arg	Leu	Pro	Ser	Pro	Met	Asp	Cys	Pro
					785					790					795
35	Ala	Ala	Leu	Tyr	Gln	Leu	Met	Leu	Asp	Cys	Trp	Gln	Lys	Asp	Arg
					800					805					810
	Asn	Ser	Arg	Pro	Lys	Phe	Asp	Asp	Ile	Val	Asn	Met	Leu	Asp	Lys
					815					820					825

Leu Ile Arg Asn Pro Ser Ser Leu Lys Thr Leu Val Asn Ala Ser  
 830 835 840  
 Ser Arg Val Ser Thr Leu Leu Ala Glu His Gly Ser Leu Gly Ser  
 845 850 855  
 5 Gly Ala Tyr Arg Ser Val Gly Glu Trp Leu Glu Ala Ile Lys Met  
 860 865 870  
 Gly Arg Tyr Thr Glu Ile Phe Met Glu Asn Gly Tyr Ser Ser Met  
 875 880 885  
 10 Asp Ala Val Ala Gln Val Thr Leu Glu Asp Leu Arg Arg Leu Gly  
 890 895 900  
 Val Thr Leu Val Gly His Gln Lys Lys Ile Met Asn Ser Leu Gln  
 905 910 915  
 Glu Met Lys Val Gln Leu Val Asn Gly Met Val Pro Val  
 920 925 928

15 (2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1839 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 20 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATTCCCCCCC GCCCTCGCCG CCGCCACCAC ACACACGCAC GCTTCTCTCC 50  
 ATCTTGATGAT TCCTTTTTTC TCCTGAACCC TCCAGTGGGG GTGCGAGTTT 100  
 GTCTTTATCA CCCCCATCC CACCGCCTTC TTTCTTCTC GCTCTCCTAC 150  
 25 CCCTCCCCAG CTTGGTGGGC GCCTCTTTCC TTTCTCGCCC CCTTTCATTT 200  
 TTATTTATTC ATATTTATTT GGC GCCCGCT CTCTCTCTGT CCCTTTCGCT 250  
 GCCTCCCTCC CTCCGGATCC CCGCTCTCTC CCCGGAGTGG CGCGTCGGGG 300  
 GCTCCGCCGC TGGCCAGGCG TGATGTTGCA CGTGGAGATG TTGACGCTGG 350  
 TGTTTCTGGT GCTCTGGATG TGTGTGTTCA GCCAGGACCC GGGCTCCAAG 400  
 30 GCCGTCGCCG ACCGCTACGC TGTCTACTGG AACAGCAGCA ACCCCAGATT 450  
 CCAGAGGGGT GACTACCATA TTGATGTCTG TATCAATGAC TACCTGGATG 500  
 TTTTCTGCCC TCACTATGAG GACTCCGTCC CAGAAGATAA GACTGAGCGC 550  
 TATGTCTCTT ACATGGTGAA CTTTGATGGC TACAGTGCCT GCGACCACAC 600  
 TTCCAAAGGG TTCAAGAGAT GGGAATGTAA CCGGCCTCAC TCTCCAAATG 650



GACCGCTGAA GTTCTCTGAA AAATTCCAGC TCTTCACTCC CTTTTCTCTA 700  
GGATTTGAAT TCAGGCCAGG CCGAGAATAT TTCTACATCT CCTCTGCAAT 750  
CCCAGATAAT GGAAGAAGGT CCTGTCTAAA GCTCAAAGTC TTTGTGAGAC 800  
CAACAAATAG CTGTATGAAA ACTATAGGTG TTCATGATCG TGTTTTTCGAT 850  
5 GTTAACGACA AAGTAGAAAA TTCATTAGAA CCAGCAGATG ACACCGTACA 900  
TGAGTCAGCC GAGCCATCCC GCGGCGAGAA CGCGGCACAA ACACCAAGGA 950  
TACCCAGCCG CTTTTTGGCA ATCCTACTGT TCCTCCTGGC GATGCTTTTG 1000  
ACATTATAGC ACAGTCTCCT CCCATCACTT GTCACAGAAA ACATCAGGGT 1050  
CTTGGAACAC CAGAGATCCA CCTAACTGCT CATCCTAAGA AGGGACTTGT 1100  
10 TATTGGGTTT TGGCAGATGT CAGATTTTGG TTTTCTTTCT TTCAGCCTGA 1150  
ATTCTAAGCA ACAACTTCAG GTTGGGGGCC TAAACTTGTT CCTGCCTCCC 1200  
TCACCCACC CCGCCCCACC CCCAGCCCTG GCCCTTGGCT TCTCTCACC 1250  
CTCCCAAATT AAATGGACTC CAGATGAAAA TGCCAAATTG TCATAGTGAC 1300  
ACCAGTGGTT CGTCAGCTCC TGTGCATTCT CCTCTAAGAA CTCACCTCCG 1350  
15 TTAGCGCACT GTGTCAGCGG GCTATGGACA AGGAAGAATA GTGGCAGATG 1400  
CAGCCAGCGC TGGCTAGGGC TGGGAGGGTT TTGCTCTCCT ATGCAATATT 1450  
TATGCCTTCT CATTCAGAAC TGTAAGATGA TCGCGCAGGG CATCATGTCA 1500  
CCATGTCAGG TCCGGAGGGG AGGTATTAAG AATAGATACG ATATTACACC 1550  
ATTTCTATA GGAGTATGTA AATGAACAGG CTTCTAAAAG GTTGAGACAC 1600  
20 TGGNTTTTTT TTTTAATATG ACTGTCTTAA AGCATTCTTG ACASCCCAAC 1650  
TTGTGCTCTC TAAAAGAAGC CTTTTTTTTT TTTCTAGGAG ACAGAGTGGG 1700  
TGTGGAATGC TAATACAGAG CAGGTGTGWA AACAGAGAAA ACTACAGGTT 1750  
TGCTGGGGGT GTGTATGTGT GAGTGCCTCT AATTTTTTTG GTGACTGGGC 1800  
AGTGCACACC AGATATTTTT TCTTTGAATA CAGATCACG 1839

25 (2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 228 amino acids
  - (B) TYPE: Amino Acid
  - (D) TOPOLOGY: Linear

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

	Met	Leu	His	Val	Glu	Met	Leu	Thr	Leu	Val	Phe	Leu	Val	Leu	Trp	
	1				5					10					15	
	Met	Cys	Val	Phe	Ser	Gln	Asp	Pro	Gly	Ser	Lys	Ala	Val	Ala	Asp	
					20					25					30	
5	Arg	Tyr	Ala	Val	Tyr	Trp	Asn	Ser	Ser	Asn	Pro	Arg	Phe	Gln	Arg	
					35					40					45	
	Gly	Asp	Tyr	His	Ile	Asp	Val	Cys	Ile	Asn	Asp	Tyr	Leu	Asp	Val	
					50					55					60	
	Phe	Cys	Pro	His	Tyr	Glu	Asp	Ser	Val	Pro	Glu	Asp	Lys	Thr	Glu	
10					65					70					75	
	Arg	Tyr	Val	Leu	Tyr	Met	Val	Asn	Phe	Asp	Gly	Tyr	Ser	Ala	Cys	
					80					85					90	
	Asp	His	Thr	Ser	Lys	Gly	Phe	Lys	Arg	Trp	Glu	Cys	Asn	Arg	Pro	
					95					100					105	
15	His	Ser	Pro	Asn	Gly	Pro	Leu	Lys	Phe	Ser	Glu	Lys	Phe	Gln	Leu	
					110					115					120	
	Phe	Thr	Pro	Phe	Ser	Leu	Gly	Phe	Glu	Phe	Arg	Pro	Gly	Arg	Glu	
					125					130					135	
	Tyr	Phe	Tyr	Ile	Ser	Ser	Ala	Ile	Pro	Asp	Asn	Gly	Arg	Arg	Ser	
20					140					145					150	
	Cys	Leu	Lys	Leu	Lys	Val	Phe	Val	Arg	Pro	Thr	Asn	Ser	Cys	Met	
					155					160					165	
	Lys	Thr	Ile	Gly	Val	His	Asp	Arg	Val	Phe	Asp	Val	Asn	Asp	Lys	
					170					175					180	
25	Val	Glu	Asn	Ser	Leu	Glu	Pro	Ala	Asp	Asp	Thr	Val	His	Glu	Ser	
					185					190					195	
	Ala	Glu	Pro	Ser	Arg	Gly	Glu	Asn	Ala	Ala	Gln	Thr	Pro	Arg	Ile	
					200					205					210	
	Pro	Ser	Arg	Leu	Leu	Ala	Ile	Leu	Leu	Phe	Leu	Leu	Ala	Met	Leu	
30					215					220					225	
	Leu	Thr	Leu													
					228											

## (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
- 35 (A) LENGTH: 37 base pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CCCTCGAGGT CGACCA YMGN GAYYTNGCNR CNMGNA 37

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 37 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCCTCGAGGT CGACCA YMGN GAYYTNGCNT GYMNA 37

10 (2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- 15 (A) LENGTH: 31 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCGCGGTGCG GCCGCCRWAN SHCCANACRT C 31

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 21 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

25 TCTGTGACAG ACGATCCTCC C 21

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 54 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GCACGGTGGA CATGTGTGAG TTTTGTCTTG GCTTTGATCA TTAGATGCTG 50

CAAC 54

## (2) INFORMATION FOR SEQ ID NO:10:

## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 54 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GTTCGAGCAT CTAATGATCA AAGCCAGGAC AAAACTCACA CATGTCCACC 50

GTGC 54

## 10 (2) INFORMATION FOR SEQ ID NO:11:

## (i) SEQUENCE CHARACTERISTICS:

- 15 (A) LENGTH: 18 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GCACTTGTAC TCCTTGCC 18

## (2) INFORMATION FOR SEQ ID NO:12:

## (i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 28 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

25 Asp Arg Tyr Ala Val Tyr Trp Asn Ser Ser Asn Pro Arg Phe Gln  
 1 5 10 15

Arg Gly Asp Tyr His Asn Asp Val Xaa Asn Asn Asp Tyr  
 20 25 28

## (2) INFORMATION FOR SEQ ID NO:13:

## (i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 24 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

35 Met Lys Thr Asn Gly Val His Asp Arg Val Phe Asp Val Asn Asp  
 1 5 10 15

Lys Val Glu Asn Xaa Leu Glu Pro Ala  
 20 24

## (2) INFORMATION FOR SEQ ID NO:14:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

5

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Val Asn Phe Asp Gly Tyr Ser Ala Xaa Asp His Thr Ser Lys Gly  
 1 5 10 15

Phe Lys Arg Xaa Glu Xaa Asn Arg  
 10 20 23

## (2) INFORMATION FOR SEQ ID NO:15:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

15

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Phe Gln Leu Phe Thr Pro Phe Ser Leu Gly Xaa Glu Xaa Arg Xaa  
 1 5 10 15

Gly Arg Glu Xaa Phe Tyr Asn Ser Xaa Ala Asn Pro  
 20 25 27

## (2) INFORMATION FOR SEQ ID NO:16:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids  
 (B) TYPE: Amino Acid  
 (D) TOPOLOGY: Linear

25

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Lys Arg Trp Glu Cys Asn Arg Pro  
 1 5 8

## (2) INFORMATION FOR SEQ ID NO:17:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 37 base pairs  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Single  
 (D) TOPOLOGY: Linear

30

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CCCTCGAGGT CGACGAYMGN TAYGCNGTNT AYTGGAA 37

## (2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 42 base pairs  
    (B) TYPE: Nucleic Acid  
    (C) STRANDEDNESS: Single  
5     (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CCGCGGTGCG GCCGCTCTAG ARTANCCRTC RAARTTNACC AT 42

What is claimed is:

1. An isolated nucleic acid encoding a functional protein having an amino acid sequence that is at least 75% homologous to the amino acid sequence shown in Figure 2 for mature human AL-1.
2. The isolated nucleic acid according to claim 1, encoding a functional protein having an amino acid  
5 sequence that is at least 85% homologous to the amino acid sequence shown in Figure 2 for mature human AL-1.
3. An isolated nucleic acid molecule comprising a nucleotide sequence encoding the amino acid sequence shown in Figure 2 for mature AL-1.
4. An isolated nucleic acid encoding a functional protein having an amino acid sequence that is at least  
10 75% homologous to the amino acid sequence of the extracellular domain shown in Figure 2 for mature human AL-1.
5. The isolated nucleic acid according to claim 4, encoding a functional protein having the amino acid sequence of the extracellular domain shown in Figure 2 for AL-1.
6. An isolated nucleic acid molecule comprising a nucleotide sequence encoding the amino acid sequence  
15 of the extracellular domain shown in Figure 2 for AL-1.
7. An isolated nucleic acid that hybridizes to DNA encoding mature human AL-1 of Figure 2 under stringent conditions, and that encodes a polypeptide comprising an immune epitope of the mature human AL-1.
8. An isolated nucleic acid comprising a nucleotide sequence encoding AL-1-IgG.
- 20 9. An expression vector comprising the nucleotide sequence of claim 1 operably linked to a promoter.
10. A host cell transformed with the expression vector of claim 9.
11. A method of using the host cell of claim 10, which comprises culturing the host cell under conditions such that the expression vector is replicated.
12. A process, comprising transforming a host cell with an expression vector of claim 9, expressing the  
25 nucleotide sequence encoding the functional protein, and culturing the cell under conditions such that the protein is synthesized.
13. The process of claim 12, further comprising recovering the polypeptide from the cell culture medium.
14. A method for producing AL-1 comprising:
  - (a) transforming a cell containing an endogenous AL-1 gene with a homologous DNA  
30 comprising an amplifiable gene and a flanking sequence of at least about 150 base pairs that is homologous with a DNA sequence within or in proximity to the endogenous AL-1 gene, whereby the homologous DNA integrates into the cell genome by recombination;
  - (b) culturing the cells under conditions that select for amplification of the amplifiable gene, whereby the AL-1 gene is also amplified; and thereafter
  - 35 (c) recovering AL-1 from the cells.
15. An isolated protein having an amino acid sequence that is at least 75% homologous to the amino acid sequence shown in Figure 2 for mature human AL-1.

16. The isolated protein according to claim 15, having an amino acid sequence that is at least 85% homologous to the amino acid sequence shown in Figure 2 for mature human AL-1.
17. The isolated protein according to claim 16, comprising the amino acid sequence shown in Figure 2 for mature AL-1.
- 5 18. An isolated protein having an amino acid sequence that is at least 75% homologous to the amino acid sequence of the extracellular domain shown in Figure 2 for mature human AL-1.
19. The isolated protein according to claim 18, having the amino acid sequence of the extracellular domain shown in Figure 2 for AL-1.
20. AL-1-IgG.
- 10 21. A pharmaceutical composition comprising the polypeptide of claim 15 and a physiologically acceptable excipient.
22. An antibody that is capable of binding to the amino acid sequence shown in Figure 2 for mature AL-1.
23. An antibody of claim 22 that is a monoclonal antibody.
24. A method of treating a neurologic disease or disorder in a mammal comprising administering a therapeutically effective amount of the composition of claim 21.
- 15 25. A method of claim 24 wherein the neurologic disease or disorder is Alzheimer's disease, amyotrophic lateral sclerosis, trauma-induced, surgery-induced, stroke-induced, ischemia-induced, infection-induced, metabolic disease-related, nutritional deficiency-induced, malignancy-induced, neurotoxicity, Bell's palsy, spinal muscular atrophy or paralysis, Parkinson's disease, epilepsy, multiple sclerosis, Huntington's chorea, Down's Syndrome, nerve deafness, Meniere's disease, post-polio syndrome, Charcot-Marie-Tooth disease, Refsum's disease, Abetalipoproteinemia, Tangier disease, Krabbe's disease, Metachromatic leukodystrophy, Fabry's disease, and Dejerine-Sottas syndrome.
- 20 26. A method of claim 24 that further comprises administering a therapeutically effective amount of NGF, BDNF, NT-3, NT-4/5, or a second eph receptor family ligand.
- 25 27. A method of diagnosing a disease or disorder of the nervous system comprising:
  - (a) contacting nucleic acid from a cell or a tissue with a labelled DNA which comprises at least 10 nucleotides of the nucleotide sequence shown in Figure 2, under conditions which will allow hybridization of complementary nucleotide sequences; and
  - (b) detecting any hybridization which occurs.
- 30 28. A method comprising:
  - (a) contacting nucleic acid from a cell or a tissue with a DNA which comprises at least 10 nucleotides of the nucleotide sequence shown in Figure 2, under conditions which will allow hybridization of complementary nucleotide sequences;
  - (b) amplifying in a polymerase chain reaction the nucleic acid to which the DNA of step (a) hybridizes; and
  - (c) detecting any nucleic acid produced by the polymerase chain reaction of step (b).
- 35 29. A method for accelerating the neovascularization of a wound which comprises applying to the wound an angiogenically effective dose of a composition comprising an AL-1 protein.



30. The method of claim 29 wherein the composition is applied topically by direct contact with the wound and the wound is a fresh surgical incision.
31. The method of claim 29 wherein the composition further comprises collagen.
32. The method of claim 29 wherein the AL-1 is AL-1-IgG.
- 5 33. The method of claim 29 wherein the wound is a burn and the composition further comprises a skin graft.
34. The method of claim 29 further comprising administering to the wound a substance selected from the group of growth factors, antibiotics, debridement agents and angiogenin.
35. A sterile surgical article comprising an AL-1 protein.
- 10 36. A composition for the debridement of burns comprising an AL-1 protein and a proteolytic enzyme which does not inactivate the neovascularizing activity of AL-1.
37. A method of modulating angiogenesis associated with a disease condition, comprising administering to a mammal an angiogenically-modulating effective amount of an AL-1 antagonist.
38. The method of claim 37, wherein the angiogenesis associated disease condition is rheumatoid arthritis or tumor formation.
- 15

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1 AGCCCCAGT CCTCTAGACA CTCTGCTTGC TTTTTCCTCCG CTTCCTCCCG CTTTTCCTCCG TTTTACGGAA ACATCATCTA GATTAAAGAT GACTCGTCTT  
 101 CTTTAAATCTC GTCCGTATCA GCACTGAAGA CTGAAAGGGA ACCTTCACCA CCACTCCAAC CCTGGTGGG GCTTAAAAA AAAAAATAAC AGTTCTAAAA  
 201 AAGAAAGGGG GACCAAAAAA CAGAAAAAGG AAAGTCTTAA GAGCAAAAGG AGCGGACTC GGGACCTCT GCAGACCTT GACTCAGCCC ACCCAGGACC  
 301 GTACTAGCCA TCCGNACTTC TAATTCTATCT TATCCAACCTG AAAGGAGGG CGGCACAAGC CAGAAGCAAA CTTCCGGCGT CTCTGCGGAT CTGTGATTCC  
 401 CACATTGAGA GGGGTCTGAGA GCCAGAAGGC ACAGGACCCC ACCAGGAGGA GGAGTTCCGC GCCTCTCTCG CCCCCTCCAC CAAGCCTGAA CCTTAGACTG  
 501 AACCACGGCG GACCTAAGAG GCAGAAGAGG GTAGTAGAAA ATG CGG GGC TCC GGG CCC CGC GGT GCG GGA CGC CGA CGG ACC CAG GGC  
 1 Met Arg Gly Ser Gly Pro Arg Gly Ala Gly Arg Arg Thr Gln Gly  
 589 AGA GGT GGC GGC GAC ACC CCC CGC GTC CCT GCC TCT CTG GCA GGC TGC TAT TCC GCA CCT CTA AAG GGG CCC CTC TGG  
 17 Arg Gly Gly Gly Asp Thr Pro Arg Val Pro Ala Ser Leu Ala Gly Cys Tyr Ser Ala Pro Leu Lys Gly Pro Leu Trp  
 670 ACG TGC CTT CTC CTG TGT GCG GCG CTC CGG ACC CTT TTG GCC AGC CCC AGT AAC GAA GTG AAT TTA TTG GAT TCG CGC ACT  
 44 Thr Cys Leu Leu Cys Ala Ala Leu Arg Thr Leu Leu Ala Ser Pro Ser Asn Glu Val Asn Leu Leu Asp Ser Arg Thr  
 751 GTC CTG GGA GAC CTT GGA TGG ATT GCT TTT CCA AAG AAT GGG TGG GAA GAG ATT GGT GAA GAT GAA AAC TAT CCC CCC  
 71 Val Leu Gly Asp Leu Gly Trp Ile Ala Phe Pro Lys Asn Gly Trp Glu Ile Gly Val Asp Glu Asn Tyr Pro Pro  
 832 ATC CAC ACC TAT CAA GTG TGC AAA GTT ATG GAA CAG AAT CAG AAT AAT TGG CTG TTG ACC AGT TGG ATC TCT AAC GAA GGT  
 98 Ile His Thr Tyr Gln Val Cys Lys Val Met Glu Gln Asn Asn Trp Leu Thr Ser Trp Ile Ser Asn Glu Gly  
 913 GCT TCC AGA ATT TTT ATT GAA CTC AAG TTT ACT CTG AGG GAT TGC AAC AGC CTT CCT GGA GGA CTG GGG ACT TGC AAG GAG  
 125 Ala Ser Arg Ile Phe Ile Glu Leu Lys Phe Thr Leu Arg Asp Cys Asn Ser Leu Pro Gly Gly Leu Gly Thr Cys Lys Glu  
 994 ACC TTT AAC ATG TAT TAT TTT GAG TCG GAT GAT GAG AAT GGG AGA AAT ATC AAA GAG AAC CAG TAC ATC AAG ATC GAT ACC  
 152 Thr Phe Asn Met Tyr Tyr Phe Glu Ser Asp Asp Glu Asn Gly Arg Asn Ile Lys Glu Asn Gln Tyr Ile Lys Ile Asp Thr  
 1075 ATT GCT GCT GAT GAG AGC TTC ACC GAA CTT GAC CTT GGA GAC CGG GTC ATG AAG CTG AAT ACG GAG GTC AGA GAT GTA GGA  
 179 Ile Ala Ala Asp Glu Ser Phe Thr Glu Leu Asp Leu Gly Asp Arg Val Met Lys Leu Asn Thr Glu Val Arg Asp Val Gly  
 1156 CCT CTG AGC AAA AAG GGA TTT TAT CTT GCT TTC CAA GAT GTC GGT GCT TGC ATC GCT CTG GTT TCT GTC CGT GTC TAC TAT  
 206 Pro Leu Ser Lys Lys Gly Phe Tyr Leu Ala Phe Gln Asp Val Gly Ala Cys Ile Ala Leu Val Ser Val Arg Val Tyr Tyr

FIG.1A

1237 AAA AAA TGT CCT TCT GTA GTT AGA CAT TTG GCT GTT TTC CCT GAC ACG ATC ACT GGA GCA GAT TCT TCC CAG TTG CTA GAG  
 233 Lys Lys Cys Pro Ser Val Val Arg His Leu Ala Val Phe Pro Asp Thr Ile Thr Gly Ala Asp Ser Ser Gln Leu Leu Glu  
 1318 GTG TCA GGC TCC TGC TGC AAC CAT TCT GTG ACA GAC GAT CCT CCC AAA ATG CAT TGC AGT GCT GAA GGG GAG TGG CTG GTT  
 260 Val Ser Gly Ser Cys Val Asn His Ser Val Thr Asp Asp Pro Pro Lys Met His Cys Ser Ala Glu Gly Glu Trp Leu Val  
 1399 CCC ATC GGG AAA TGC ATG TGC AAG GCC GGA TAT GAA GAG AAA AAT GGT ACC TGT CAA GTG TGC AGA CCT GGG TTC TTC AAA  
 287 Pro Ile Gly Lys Cys Met Cys Lys Ala Gly Tyr Glu Glu Lys Asn Gly Thr Cys Gln Val Cys Arg Pro Gly Phe Phe Lys  
 1480 GCC TCT CCT CAC AGC CAG ACC TGC AGC AAA TGT CCA CCT CAC AGT TAC ACC CAT GAG GAA GCT TCC ACC TCT TGT GTC TGT  
 314 Ala Ser Pro His Ser Gln Thr Cys Ser Lys Cys Pro Pro His Ser Tyr Thr His Glu Glu Ala Ser Thr Ser Cys Val Cys  
 1561 GAA AAG GAT TAT TTC AGG AGG GAA TCT GAT CCG CCC ACA ATG GCA TGC ACA ACT CCT TCT CCA GTC ACC AAC GTG AAG AAG  
 341 Glu Lys Asp Tyr Phe Arg Arg Glu Ser Asp Pro Pro Thr Met Ala Cys Thr Thr Pro Ser Pro Val Thr Asn Val Lys Lys  
 1642 GGG AAG ATC GCA AAG AAC AGC ATT TCT TTG TCT TGG CAA GAG CCA GAT CGC CCC AAT GGG ATC ATC CTG GAG TAC GAA ATC  
 368 Gly Lys Ile Ala Lys Asn Ser Ile Ser Leu Ser Trp Gln Glu Pro Asp Arg Pro Asn Gly Ile Ile Leu Glu Tyr Glu Ile  
 1723 AAG TAC TTT GAA AAG GAC CAA GAG ACC AGC TAC ACA ATT ATC AAG TCT AAA GAG ACC ACT ATT ACG GCA GAG GGC CTG AAA  
 395 Lys Tyr Phe Glu Lys Asp Gln Glu Thr Ser Tyr Thr Thr Ile Ile Lys Ser Lys Glu Thr Thr Ile Thr Ala Glu Gly Leu Lys  
 1804 CCT GCG TCT GTG TAT GTC TTC CAA ATT CGA GCA CGT ACA GCA GCA GGC TAC GGC GTC TTC AGT CGA CGG TTT GAG TTT GAA  
 422 Pro Ala Ser Val Tyr Val Phe Gln Ile Arg Ala Arg Thr Ala Ala Gly Tyr Gly Val Phe Ser Arg Arg Phe Glu Phe Glu  
 1885 ACC ACA CCA CCA GTT TCA GTT GCA GCA TCT AAT GAT CAA AGC CAG ATT CCC ATC ATT GCG GTG TCA GTG ACG GTG GGA GTC ATC  
 449 Thr Thr Pro Val Ser Val Ala Ala Ser Asn Asp Gln Ser Gln Ile Pro Ile Ile Ala Val Ser Val Thr Val Gly Val Ile  
 1966 TTG TGC GCA GTG ATG ATC GGC TTC CTC CTC AGT GGC AGT TGC TGC GAA TGT GGC TGT GGC TGT TCC CTG TGC GCT  
 476 Leu Leu Ala Val Met Ile Gly Phe Leu Leu Ser Gly Ser Cys Cys Glu Cys Gly Arg Ala Ser Ser Leu Cys Ala  
 2047 GTT GCC CAT CCA AGC CTA ATA TGG CGG TGT GGC TAC AGC AAA GCA AAG CAG GAT CCA GAA GAG GAA AAG ATG CAC TTT CAT  
 503 Val Ala Ala His Pro Ser Leu Ile Trp Arg Cys Gly Tyr Ser Lys Ala Lys Gln Asp Pro Glu Glu Glu Lys Met His Phe His  
 2128 AAC GGG CAC ATT AAA CTG CCA GGA GTA AGA ACG TAC ATT GAT CCA CAC ACC TAC GAA GAT CCC ACT CAA GCC GTT CAT GAA  
 530 Asn Gly His Ile Lys Leu Pro Gly Val Arg Thr Tyr Ile Asp Pro His Thr Tyr Glu Asp Pro Thr Gln Ala Val His Glu  
 2209 TTT GCC AAG GAG ATC GAG GCC TCA TGC ATC ACC ATT GAG AGA GTT ATT GGA GCA GGT GAA TTT GGT GAA GTT TGT AGT GGA  
 557 Phe Ala Lys Glu Ile Glu Ala Ser Cys Ile Thr Ile Glu Arg Val Ile Gly Ala Glu Phe Gly Glu Val Cys Ser Gly

FIG. 1B

2290 CGT TTG AAA CTA CCC GGA AAA AGA GAA TTG CCT GTG GCT ATC AAA ACT CTT AAA GTA GGC TAC ACT GAA AAG CAG CGC AGA  
 584 Arg Leu Lys Leu Pro Gly Lys Arg Glu Leu Pro Val Ala Ile Lys Thr Leu Lys Val Gly Tyr Thr Glu Lys Gln Arg Arg  
 2371 GAT TTT CTG AGT GAA GCG AGT ATC ATG GGG CAG TTT GAT CAT CCA AAC ATC ATC CAT CTA GAA GGC GTT GTG ACT AAA AGT  
 611 Asp Phe Leu Ser Glu Ala Ser Ile Met Gly Gln Phe Asp His Pro Asn Ile Ile His Leu Glu Gly Val Val Thr Lys Ser  
 2452 AAA CCT GTG ATG ATA GTG ACA GAG TAC ATG GAG AAT GGC TCC TTA GAC ACA TTT TTA AAG AAA AAC GAT GGC CAG TTC ACT  
 638 Lys Pro Val Met Ile Val Thr Glu Tyr Met Glu Asn Gly Ser Leu Asp Thr Phe Leu Lys Lys Asn Asp Gly Gln Phe Thr  
 2533 GTG ATT CAG CTT GTT GGC ATG CTG AGA GGC ATC GCT GCA GGA ATG AAG TAC TCT TCT GAC ATG GGC TAC GTG CAC AGA GAC  
 665 Val Ile Gln Leu Val Gly Met Leu Arg Gly Ile Ala Ala Gly Met Lys Tyr Leu Ser Asp Met Gly Tyr Val His Arg Asp  
 2614 CTT GCT GCT AGA AAC ATC TTA ATC AAC AGT AAC CTT GTG TGC AAA GTG TCT GAC TTT GCA CTT TCC AGG GTG CTG GAA GAT  
 692 Leu Ala Ala Arg Asn Ile Leu Ile Asn Ser Asn Leu Val Cys Lys Val Ser Asp Phe Gly Leu Ser Arg Val Leu Glu Asp  
 2695 GAT CCT GAG GCA GCC TAT ACC ACA AGG GGA GGC AAA ATT CCA ATC AGG TGG ACT GCT CCA GAA GCA ATA GCT TTT CGA AAG  
 719 Asp Pro Glu Ala Ala Tyr Thr Thr Arg Gly Gly Lys Ile Pro Ile Arg Trp Thr Ala Pro Glu Ala Ile Ala Phe Arg Lys  
 2776 TTT ACC TCT GCC AGT GAT GTC TGG AGC TAT GGA ATC GTA ATG TGG GAA GTT GTG TCC TAT GGA GAG AGA CCG TAC TGG GAG  
 746 Phe Thr Ser Ala Ser Asp Val Trp Ser Tyr Gly Ile Val Met Trp Glu Val Ser Tyr Gly Glu Arg Pro Tyr Trp Glu  
 2857 ATG ACC AAT CAG GAT GTG ATC AAG GCA GTG GAA GGC TAC CGC CTG CCA AGC CCC ATG GAT TGT CCT GCT GCC CTC TAT  
 773 Met Thr Asn Gln Asp Val Ile Lys Ala Val Glu Glu Gly Tyr Arg Leu Pro Ser Pro Met Asp Cys Pro Ala Ala Leu Tyr  
 2938 CAA TTA ATG CTG GAT TGC TGG CAG AAA GAT CGC AAC AGC AGG CCC AAG TTT GAT GAC ATA GTC AAC ATG CTG GAT AAG CTG  
 800 Gln Leu Met Leu Asp Cys Trp Gln Lys Asp Arg Asn Ser Arg Pro Lys Phe Asp Ile Val Asn Met Leu Asp Lys Leu  
 3019 ATA CGA AAC CCA AGT AGT TTG AAG ACA CTG GTG AAC GCG TCA AGC AGG GTA TCT ACT TTG TCG GCA GAA CAT GGA TCT TTG  
 827 Ile Arg Asn Pro Ser Ser Leu Lys Thr Leu Val Asn Ala Ser Ser Arg Val Ser Thr Leu Leu Ala Glu His Gly Ser Leu  
 3100 GGG TCT GGG GCC TAC AGA TCA GTA GGT GAA TGG CTA GAA GCA ATC AAA ATG GGC CGG TAC ACA GAG ATT TTC ATG GAA AAT  
 854 Gly Ser Gly Ala Tyr Arg Ser Val Gly Glu Trp Leu Glu Ala Ile Lys Met Gly Arg Tyr Thr Glu Ile Phe Met Glu Asn  
 3181 GGA TAC AGT TCA ATG GAC GCT GTG ACC TTG GAG GAT TTG AGA CGC CTG GGA GTG ACT CTG GTC GGT CAC CAG  
 881 Gly Tyr Ser Ser Met Asp Ala Val Ala Gln Val Thr Leu Glu Asp Leu Arg Arg Arg Leu Thr Leu Val Gly His Gln

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FIG.1C

3262 AAG AAG ATC ATG AAC AGC CTT CAA GAG ATG AAG GTG CAG CTG GTA AAC GGG ATG GTG CCA GTG TGA CCC GCACACGGGT  
 908 Lys Lys Ile Met Asn Ser Leu Gln Glu Met Lys Val Gln Leu Val Asn Gly Met Val Pro Val OP\*  
 3341 CACITTCCTCA AGTGAACAAC TCTGCACITTT GTAAACAGCC CTAAGATTTA TTTTAAACAGA GAAAGGGAAA TGGGTGGTTC CTAAACCTTTT GAAGGCACIT  
 3441 GTCTCATCCT TTGACTTATA ATCAACATTT TACTAAAATC TCCAGATCCTT CTTCTTAAATG TCTTCGTTTTT TTTCACCTATG TAAATGTAAC CTGCGAAGAG  
 3541 AGCTAACATG AGAAACAACA TCCTATAAAA ACATAGTAAC TAAATCTTTC TCCACTTGCA GCCCCTTTTCA AAACCTACCAG GGATCGACTT GAAAGGAAAA  
 3641 GTTTTAAAGC CATGTGTGGG CAAAGAAAACG CTGCATTTTA CTGACGTTTA CTTGAGGTTT TATTTGTCTG CATAAGTGTA TTGGAGAGCA ATATGATTAG  
 3741 ATTATTTCTT AAATACAGTT TGTAAATTAA AATGGAATTA CATGTTATAA GTTATAGAAA ATAGTTTACA GACATGTTGC CCGGTCAAGG AAAAGTTCAG  
 3841 CACAGGGTGT ATATTTATTT TTCTGTGTTA TATAATTTAC TTTTAGTTGC ACTTCTAGAG AGTATTAGGC AATGAATGTG TATAGACTGT ATAGTTTGCA  
 3941 ATATACCGAG GAATGGACTT AAATTGGAAA TGTATGTATA TGTGTGTGTA TGTGCGTATG TGTGTGTGTG TTTTGAATATG TAGATGGTAT TGTTCCTGCTT  
 4041 GCCTTTGTG TAGGGTTTTA ATTTTGGCCT CATAACAGCA AGGGTGTCTT AGACTATTTT ATGGGTAAGA GGAATAGGAA GCCTTAGACC AAATTTCCCT  
 4141 CAAGTAGGTG TCCTTTCTCT CATTT

FIG.1D

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1 ATTCCCCCGCCCTCGCCGCCGCCACCACACACACGCACGCTTCTCTCCATCTTGTGAT  
61 TCCTTTTTCCTCCTGAACCTCCAGTGGGGGTGCGAGTTTGTCTTTATACCCCCATCC  
121 CACCGCCTTCTTTTCTCTCGCTCTCCTACCCCTCCCCAGCTTGGTGGGCGCCTCTTTCC  
181 TTTCTGCCCCCTTTTCATTTTTATTTATTCATATTTATTTGGCGCCCGCTCTCTCTCTGT  
241 CCGTTTGCTGCCCTCCCTCCCTCCGGATCCCCGCTCTCTCCCCGGAGTGGCGCGTGGGG  
301 GCTCCGCCGCTGGCCAGGCGTGATGTTGCACGTGGAGATGTTGACGCTGGTGTCTTCTGGT  
1 M L H V E M L T L V F L V  
361 GCTCTGGATGTGTGTGTTTCAGCCAGGACCCGGGCTCCAAGGCCGTGCGCGACCGCTACGC  
14 L W M C V F S Q D P G S K A V A D R Y A  
421 TGTCTACTGGAACAGCAGCAACCCAGATTCCAGAGGGGTGACTACCATATTGATGTCTG  
34 V Y W N S S N P R F O R G D Y H I D V C  
481 TATCAATGACTACCTGGATGTTTTCTGCCCTCACTATGAGGACTCCGTCCCAGAAGATAA  
54 I N D Y L D V F C P H Y E D S V P E D K  
541 GACTGAGCGCTATGTCCTCTACATGGTGAACCTTTGATGGCTACAGTGCCTGCGACCACAC  
74 T E R Y V L Y M V N F D G Y S A C D H T  
601 TTCAAAGGGTTCAAGAGATGGGAATGTAACCGGCCTCACTCTCAAATGGACCGCTGAA  
94 S K G F K R W E C N R P H S P N G P L K  
661 GTTCTCTGAAAAATTCCAGCTCTTCACTCCCTTTTCTCTAGGATTTGAATTCAGGCCAGG  
114 F S E K F O L F T P F S L G F E F R P G  
721 CCGAGAATATTTCTACATCTCCTCTGCAATCCCAGATAATGGAAGAAGGTCCTGTCTAAA  
134 R E Y F Y I S S A I P D N G R R S C L K  
781 GCTCAAAGTCTTTGTGAGACCAACAATAGCTGTATGAAAATATAGGTGTTTCATGATCG  
154 L K V F V R P T N S C M K T I G V H D R  
841 TGTTCCTGATGTTAACGACAAAGTAGAAAATTCATTAGAACCAGCAGATGACACCGTACA  
174 V F D V N D K V E N S L E P A D D T V H  
901 TGAGTCAGCCGAGCCATCCCGCGGCGAGAACGCGGCACAAACACCAAGGATACCCAGCCG  
194 E S A E P S R G E N A A Q T P R I P S R  
961 CCTTTTGGCAATCCTACTGTTCTCTGCGATGCTTTTGGACATTATAGCACAGTCTCCT  
214 L L A I L L F L L A M L L T L O  
1021 CCCATCACTTGTACAGAAAACATCAGGGTCTTGGAACACCAGAGATCCACCTAACTGCT  
1081 CATCCTAAGAAGGGACTTGTTATTGGGTTTTGGCAGATGTCAGATTTTGGTTTTCTTTCT  
1141 TTCAGCCTGAATTCTAAGCAACAACCTTCAGGTTGGGGCCTAAACTTGTCTCTGCCCTCCC  
1201 TCACCCACCCCGCCCCACCCCGAGCCTGGCCCTTGGCTTCTCTACCCCTCCCAAATT  
1261 AAATGGACTCCAGATGAAAATGCCAAATTGTCATAGTGACACCAGTGGTTCGTCAGCTCC  
1321 TGTGCATTCTCCTCTAAGAACTCACCTCCGTTAGCGCACTGTGTCAGCGGGCTATGGACA  
1381 AGGAAGAATAGTGGCAGATGCAGCCAGCGCTGGCTAGGGCTGGGAGGGTTTTGCTCTCCT  
1441 ATGCAATATTTATGCCTTCTCATTGAGAACTGTAAGATGATCGCGCAGGGCATCATGTCA  
1501 CCATGTCAGGTCCGGAGGGGAGGTATTAAGAATAGATACGATATTACCAATTTCTATA  
1561 GGAGTATGTAATGAACAGGCTTCTAAAAGGTTGAGACACTGGNTTTTTTTTTTAATATG  
1621 ACTGTCTTAAAGCATTCTTGACASCCCAACTTGTGCTCTCTAAAAGAAGCCTTTTTTTT  
1681 TTTCTAGGAGACAGAGTGGGTGTGGAATGCTAATACAGAGCAGGTGTGWAACAGAGAAA  
1741 ACTACAGGTTTGCTGGGGGTGTGTATGTGTGAGTGCCTCTAATTTTTTTGGTGACTGGGC  
1801 AGTGCACACCAGATATTTTTTCTTTGAATACAGATCACG

FIG. 2

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AL-1 1 M L H V E - - M L T L V F L V L W M C V F S Q D P G S K A V A D R R Y A V Y W N S S N P R F O R - - -  
 EII 1 M A P A O R P L L P L L L L L P L R A R N E D P - A R A N A A D R R Y A V Y W N S S N P R F O V S A V  
 B61 1 - - - - - M E F L W A P L L G L C C - - - - S L A A A A D R R H T V F W N S S N P K F R N - - -  
 EHK1-L 1 M A A P L L L L L L V P V P L L P L L A O G P G G - A L G N R H A V Y W N S S N Q H L R R - - -  
 Lerk2ECD 1 M A R P G O R W L G K W L V A M V V M A L C R L A - T P L A K N L E P V S W S S L N P K F L S - - -

AL-1 46 - - - G D Y H I D V C I N D Y L D V F C P H Y - - - E D S V - P E D K T E R Y V L Y M V N F D G Y S  
 EII 50 G D G G Y T V E V S I N D Y L D I Y C P H Y - - - G A P L P P A E R M E R Y I L Y M V N G E G H A  
 B61 35 - - - E D Y T I H V Q L N D Y Y D I I C P H Y - - - E D H S V A D A A M E Q Y I L Y L V E H E E Y Q  
 EHK1-L 47 - - - E G Y T V O V N V N D Y L D I Y C P H Y N S S G A G P G P G G G A E Q Y V L Y M V S R N G Y R  
 Lerk2ECD 47 - - G K G L V I Y P K I G D K L D I I C P R - - - - A E A G R P Y E Y Y K L Y L V R P E Q A A

AL-1 89 A C D H T S K G F K R W E C N R P H S P N G P L K F S E K F O L F T P F S L G F E F R P G R E Y F Y  
 EII 97 S C D H R O R G F K R W E C N R P A A P G G P L K F S E K F O L F T P F S L G F E F R P G H E Y Y Y  
 B61 79 L C Q P O S K D O V R W Q C N R P S A K H G P E K L S E K F O R F T P F T L G K E F K E G H S Y Y Y  
 EHK1-L 94 T C - N A S Q G F K R W E C N R P H A P H S P I K F S E K F O R Y S A F S L G Y E F H A G H E Y Y Y  
 Lerk2ECD 88 A C S T V L D P N V L V T C N R P - - - E Q E I R F T I K F O E F S P N Y M G L E F K K H H D Y Y I

FIG. 3A

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AL-1 139 T S S - - - A I P D N G R R S - - - C - - - L K L K V F V R P T N S C M K T I G V H D R V - - F D V N  
 EII 147 I S A - - - T P P N L V D R P - - - C - - - L R L K V Y V R P T - - - N E T L - - - Y E - -  
 B61 129 I S K - - - P I H Q H E D R - - - C - - - L R L K V T V S G K I T - - - H S P Q A H V - - -  
 EHK1-L 143 I S T - - - P T H N L - H M K - - - C - - - L R M K V F V C C A S T S H S G E K P V P T L P Q F T M G  
 Lerk2ECD 135 T S T S N G S L E G L E N R E G G V C R T R T M K I I M K V G O D P N A V T P E O L I T S R P S K E

AL-1 179 D K V E - N S L E P A D D T V H E S A E P S R G E N A A O T P R I P S R L - L A I L L F - L L A M L  
 EII 176 - - - - - A P E P I F T S N S S C S G L G G C H L - - - - F - L T T V P V - L W S L L  
 B61 160 - - - - - N P Q E K R L A A D D P E V R V L H S I G H S A A P R L - F P L A M T V L L L - P L L L L  
 EHK1-L 184 P M V K I M V L E D F E G E N P Q V P K L E K S I S G T S P K R E H L P L A V G I A F F - L M T F L  
 Lerk2ECD 185 A D M T V K M A T O A P G S R G S L G D S D G K H E T V N Q E E K S G P G A S G G S S G D P D G F F

AL-1 226 L T L  
 EII 208 G S -  
 B61 203 Q T P  
 EHK1-L 233 A S -  
 Lerk2ECD 235 N S K

FIG. 3B



## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 95/14016

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C07K14/47 C12N15/12 C07K16/18 C07K19/00 C12N05/10  
C12Q1/68 A61K38/12

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CELL, 1994, 79 157-168, CHENG HJ ET AL 'IDENTIFICATION AND CLONING OF ELF-1, A DEVELOPMENTALLY EXPRESSED LIGAND FOR THE MEK4 AND SEK RECEPTOR TYROSINE KINASES' see the whole document ---	1-22,27, 35,36
Y	ONCOGENE, vol. 8, 1993 pages 3277-3288, XP 000566895 MAISONPIERRE P.C. ET AL. 'Ehk-1 and Ehk-2: two novel members of the Eph receptor-liketyrosine kinase family with distinctive structures and neuronal expression.' see the whole document --- -/--	1-22,27, 35,36

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

- 'A' document defining the general state of the art which is not considered to be of particular relevance
- 'E' earlier document but published on or after the international filing date
- 'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- 'O' document referring to an oral disclosure, use, exhibition or other means
- 'P' document published prior to the international filing date but later than the priority date claimed

- 'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- 'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- 'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- '&' document member of the same patent family

Date of the actual completion of the international search

26 March 1996

Date of mailing of the international search report

12 APRIL 1996

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. NEUROSCI. RES. 37:129-143(01-1994).. ZHOU R. ET AL. "Isolation and characterization of Bsk, a growth factor receptor-like tyrosine kinase associated with the limbic system' see the whole document ---	1-22,27, 35,36
Y A	EP,A,0 597 503 (AMGEN INC) 18 May 1994 see the whole document ---	27,35,36 1-22
A	WO,A,94 11384 (IMMUNEX CORP) 26 May 1994 see the whole document ---	1-22
P,X	WO,A,95 27060 (REGENERON PHARMA) 12 October 1995 see claim 10 ---	1-19
P,X	NEURON, vol. 14, May 1995 pages 973-981, WINSLOW J.W. ET AL. 'Cloning of AL-1, a ligand for an Eph-related tyrosine kinase receptor involved in axon bundle formation' see the whole document ---	1-19
X,P	SCIENCE, vol. 266, November 1994 pages 816-819, DAVIS S. ET AL. 'LIGANDS FOR EPH RELATED RECEPTOR TYROSINE KINASES THAT REQUIRE MEMBRANE ATTACHMENT OR CLUSTERING FOR ACTIVITY' see the whole document -----	1-19

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/14016

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 24,25,29-34,37,38 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

Information on patent family members

Original Application No

PCT/US 95/14016

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0597503	18-05-94	AU-B- 5599794	08-06-94
		CN-A- 1094446	02-11-94
		FI-A- 952328	10-07-95
		NO-A- 951861	11-07-95
		WO-A- 9411020	26-05-94
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WO-A-9411384	26-05-94	AU-B- 5667594	08-06-94
		CA-A- 2148484	26-05-94
		EP-A- 0669929	06-09-95
		NZ-A- 258697	26-03-96
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WO-A-9527060	12-10-95	AU-B- 2278995	23-10-95
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